FACULDADE DE ARQUITETURA
UNIVERSIDADE DE LISBOA

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TÉCNICO LISBOA

MULTIPLE-STRESSORS IMPACTS ON MEDITERRANEAN STREAM COMMUNITIES: RESPONSES TO WATER SCARCITY AND WATER POLLUTION

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THESIS PRESENTED TO OBTAINED THE DOCTOR DEGREE IN
RIVER RESTORATION AND MANAGEMENT

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Para o meu Pai

Sometimes, if you stand on the bottom rail of a bridge and lean over to watch the river slipping slowly away beneath you, you will suddenly know everything there is to be known.

(A. A. Milne *in The House at Pooh Corner*)

IMPACTO DE PRESSÕES MÚLTIPLAS EM COMUNIDADES RIBEIRINHAS MEDITERRÂNICAS: RESPOSTAS À ESCASSEZ HÍDRICA E À POLUIÇÃO AQUÁTICA

RESUMO

Os ecossistemas ribeirinhos são fortemente afetados por múltiplos stresses de origem antropogénica e natural originando efeitos combinados com interações complexas devido a sinergias e antagonismos e, portanto, representam um desafio acrescido para prever alterações ecológicas e desenvolver estratégias eficazes de gestão e restauro.

Nesta investigação, foram avaliadas diversas respostas (fisiológicas, comportamentais e funcionais) de importantes elementos biológicos ribeirinhos (biofilmes e macroinvertebrados) ao impacto de stresses que frequentemente interagem em rios Mediterrânicos, relacionados com escassez hídrica e poluição aquática, tais como a redução da velocidade do caudal, efeito da estagnação do fluxo (intermitência), contaminação orgânica/nutrientes e consequente hipoxia.

Na primeira parte, os efeitos da redução de velocidade de caudal e depleção de oxigénio dissolvido foram testados em macroinvertebrados a nível comportamental (i.e., *drift*) e funcional (i.e., *traits*), resultando, de um modo geral, em alterações dos padrões de *drift* e na perda da diversidade de *traits* com potenciais impactos no funcionamento e serviços do ecossistema. Numa segunda parte, foram investigados os efeitos da estagnação do fluxo de água e da contaminação de um efluente em biofilmes e nos seus consumidores, mostrando um efeito inibitório generalizado das respostas biológicas sob o efeito de estagnação e um aumento sob o efeito do efluente. O efeito combinado destes stresses induziu alterações nas proporções de microrganismos constituintes dos biofilmes (i.e., diminuição da diversidade de bactérias e algas e aumento de fungos).

Na última parte, foram monitorizadas variações hidrológicas e na qualidade da água num ribeiro urbano ao longo do tempo, observando-se uma comunidade de macroinvertebrados empobrecida com baixa diversidade e elevada abundância de grupos tolerantes e resistentes, associado a um padrão de diversos stresses a atuar em conjunto.

A gestão em rios mediterrânicos deverá priorizar a redução das descargas de efluentes e reforçar o tratamento de águas residuais, especialmente durante as épocas de menor caudal.

Palavras-chave: Stresses-múltiplos, Mesocosmos, Biofilme, Macroinvertebrados, Escassez hídrica, Água residual

ABSTRACT

Freshwater ecosystems are influenced by diverse anthropogenic stressors and natural disturbances producing interacting effects (synergies and antagonisms) often unpredicted based on their independent effects, and thereby represent a major concern to predict ecological changes and develop effective management and restoration strategies. The aim of this *Thesis* was to assess different responses of key stream biological elements (i.e., biofilms and macroinvertebrates) to the impact of main stressors acting in concert in Mediterranean rivers related with water scarcity and pollution (i.e., flow velocity reduction/stagnation and sewage contamination/hypoxia), using mesocosm approaches and evidences of a case study. In a first part, the effects of flow reduction and oxygen depletion conditions in two seasons were evaluated in macroinvertebrates behavioural and functional responses, conducting to evidences of changes in drift patterns and loss of trait diversity with potential impacts on ecosystem functioning and services.

In a second part was investigated how single and combined effects of water flow stagnation and sewage contamination affect primary producers and primary consumers, showing that while stagnant water had an overall inhibitory effect, sewage inputs increased biological responses. Also, stressors combined effect revealed to be unfavourable for some biofilm assemblages (decreasing bacteria and algae diversity) while advantageous to other (increasing fungi diversity).

In the last part, flow and water quality variations were followed over time in a Mediterranean urban stream, leading to observations of an impoverished community with low diversity and high abundance of tolerant taxa and resistance traits, which was associated with multiple-stressors patterns of urban ecosystems.

Overall it was showed that the effects of representative multiple-stressor scenarios on Mediterranean stream biota are dependent on water scarcity conditions and that reductions on sewage inputs needs to be given priority by stream managers to prevent further degradation of these aquatic ecosystems.

Keywords: Multiple-stressors, Mesocosm, Biofilm, Macroinvertebrates, Flow depletion, Sewage

RESUMO ALARGADO

O principal foco desta tese assenta na crescente evidência de que a grande maioria dos ecossistemas de água doce estão continuamente expostos a múltiplas pressões de origem antropogénica assim como perturbações naturais. A combinação destas pressões, que operam em simultâneo, tem o potencial de gerar interações com efeitos antagonísticos e sinergísticos, afetando de forma imprevisível as comunidades aquáticas, processos ecossistémicos, a biodiversidade, e em última análise, o funcionamento do ecossistema. Compreender o impacto de pressões múltiplas, torna-se assim, de extrema importância para identificar, prever e prevenir a deterioração dos ecossistemas ribeirinhos e fornecer informações úteis para as práticas de gestão e restauro ecológico.

Os rios e ribeiros são particularmente suscetíveis aos impactos humanos, os quais causam efeitos diretos na disponibilidade hídrica e na sua qualidade ecológica e físico-química. A poluição aquática de origem difusa e pontual resultante da crescente atividade urbana, industrial e agrícola constituiu uma das pressões mais recorrentes nestes sistemas aquáticos. Isto torna-se particularmente preocupante nas regiões do Mediterrâneo. Nos rios mediterrânicos, a flutuação hidrológica sazonal que comporta períodos naturais de redução extrema do caudal (frequentemente reduzido a poças ou seca total), aumento da captação de água para atividades humanas aliados a alterações globais resultam em períodos de escassez hídrica graves, com efeitos diretos e indiretos sobre outros fatores de stress como a descarga de efluentes domésticos e industriais que contribuem para a degradação da qualidade da água com elevadas cargas orgânicas, nutrientes e condições de hipoxia entre outros. Estes efeitos podem, portanto, ser amplificados devido à baixa capacidade de diluição destes sistemas, interagindo de formas complexas e difíceis de antever devido a potenciais sinergismos e antagonismos com efeitos significantes nas comunidades ribeirinhas envolvendo diferentes níveis da cadeia trófica como produtores primários (i.e., biofilmes) e consumidores primários (i.e., macroinvertebrados). Como tal, sob o efeito de pressões múltiplas são esperadas alterações quer a nível funcional, quer a nível estrutural nas comunidades lóticas mediterrânicas, em particular sob o efeito combinado da diminuição do fluxo de água e da contaminação com efluentes de águas residuais.

Posto isto, esta dissertação tem como objetivo global contribuir com avanços no conhecimento relativo ao efeito de pressões múltiplas atendendo a diversas respostas de diferentes elementos biológicos de grande importância em rios e ribeiros mediterrânicos, e é assim, composta por um conjunto de estudos direcionados para o efeito das interações de stresses bastante comuns em sistemas aquáticos mediterrânicos diretamente relacionadas com a escassez hídrica e a poluição aquática.

De acordo com a dificuldade em compreender e antever os efeitos interativos das pressões, anteriormente mencionado, torna-se crucial o desenvolvimento de estudos com aplicação de metodologias eficazes e experiências apropriadas que promovam a compreensão do modo de interação das pressões múltiplas, acedendo à importância relativa de cada pressão e de como estas operam em conjunto no ecossistema. No entanto, esses tipos de estudos são escassos em sistemas naturais, muito provavelmente devido a todas as dificuldades que estão envolvidas na manipulação de mais que uma pressão num ambiente experimental natural e realístico. Experiências manipulativas como uso de desenhos fatoriais em sistemas de mesocosmos possibilitam o controlo de tratamentos onde são aplicados determinados níveis de pressões para detetar e quantificar os seus efeitos em recetores específicos. Assim, o uso de uma abordagem em mesocosmos foi usada na maioria dos estudos que compõem esta tese, na qual o principal objetivo implica a avaliação dos efeitos simples e combinados dos diferentes stresses.

Esta investigação dividiu-se em 3 partes principais. Numa primeira parte, foram abordados os efeitos da diminuição da velocidade da corrente e da diminuição do oxigénio dissolvido na água (como resultado da atividade microbiana de cargas orgânicas) nas respostas comportamentais (drift) e funcionais (traits) da comunidade de macroinvertebrados. Aqui, foram enquadrados os objetivos específicos: (i) avaliar as diferenças entre o comportamento de "drift" da comunidade de invertebrados sob o efeito independente e sob o efeito combinado da redução de fluxo e da depleção de oxigénio; (ii) avaliar o efeito de alterações sazonais nas respostas da comunidade aos efeitos das pressões; (iii) determinar as alterações na composição funcional dos macroinvertebrados (traits) após exposição aos fatores de stress e, (iv) avaliar quais as características funcionais (traits) que melhor revelam o efeito independente ou combinado dos fatores de stress. No Capítulo 2, foi verificado que apesar da redução de fluxo e da depleção de oxigénio terem atuado independentemente em direções opostas (diminuindo e aumentando o comportamento de drift, respetivamente), o seu efeito combinado resultou num aumento sinergístico do drift da comunidade, ou seja, as condições de depleção de oxigénio alteraram o efeito da redução de fluxo, aumentando efetivamente o drift mais do que seria esperado com base nos seus efeitos independentes. Foram também encontradas evidências da variação

sazonal dos padrões de drift, os quais podem depender da fase do ciclo de vida dos indivíduos e do seu comportamento sazonal. Verificou-se ainda, no Capítulo 3, que a reduzida velocidade do fluxo e a falta de oxigénio dissolvido podem induzir uma perda da diversidade funcional nas comunidades de macroinvertebrados, o que pode resultar em variações no funcionamento do ecossistema através, por exemplo, de alterações locais na biomassa, produção secundária, metabolismo, bem como resultando em perdas de biodiversidade ou alterações dos padrões de distribuição dos organismos. A utilização de alguns *traits* poderá ser um potencial contributo para detetar alterações específicas no ecossistema.

Na segunda parte, foi investigado o efeito independente e combinado da estagnação de água (efeito de poça) e da contaminação de água residual (efluente) em produtores primários (biofilmes) e consumidores primários (macroinvertebrados raspadores herbívoros "grazers"), tendo-se estabelecido como objetivos principais: (i) avaliar respostas estruturais dos biofilmes ao efeito dessas pressões; (ii) investigar se esses efeitos são sentidos no nível trófico seguinte, através de efeitos diretos sobre os "grazers" e efeitos indiretos sobre a qualidade dos biofilmes como recurso alimentar e, (iii) investigar alterações nas proporções das comunidades microbianas que compõem o biofilme sob os efeitos independentes e combinados das pressões antropogénicas, incluindo também uma pressão natural (i.e., herbivoria), especificamente avaliando a abundancia relativa de bactérias, fungos e algas associadas ao biofilme utilizando uma abordagem molecular. No capítulo 4, verificou-se um efeito independente da contaminação orgânica/nutrientes num aumento da biomassa de biofilme e concentração de clorofila, aumentando também o consumo de oxigénio e a atividade alimentar por parte dos "grazers". Combinando os stresses, o efeito que a contaminação de água residual demonstrou no aumento na biomassa de biofilme e na atividade alimentar, foi reduzida pelo "efeito de poça" (fator antagonista). Os resultados, sugerem que a contaminação tem um efeito direto sobre os produtores primários e um efeito indireto nos seus consumidores, efeito esse que é influenciado pela estagnação do fluxo de água. No capítulo 5, dados moleculares ajudaram a detetar alterações na proporção das comunidades microbianas do biofilme em resposta à combinação dos stresses, demonstrando que a diversidade de algas e bactérias poderá ser negativamente afetada, enquanto a diversidade de fungos poderá ser positivamente aumentada.

Na terceira e última parte, foi usada uma abordagem diferente com o estudo de um caso específico (*case-study*), investigando o efeito de variações na quantidade e na qualidade da água na comunidade de macroinvertebrados com a monitorização de um ribeiro urbano mediterrânico ao longo do tempo. Neste estudo, os principais objetivos foram: (i) analisar o efeito das alterações hidrológicas (caudal, precipitação) e de parâmetros físico-químicos da água (nutrientes, condutividade, velocidade do fluxo, etc.) nas comunidades de macroinvertebrados

a nível estrutural, funcional (*traits*) e na qualidade biológica (índices biológicos) e, (ii) testar a utilização de *taxa* específicos e abundantes em ribeiros urbanos (Chironomidae) identificados em níveis mais elevados de resolução taxonómica e respetivas características biológicas, como uma ferramenta mais especializada para detetar alterações na qualidade dos ecossistemas lóticos urbanos sob múltiplos fatores de stress.

Finalmente, foram discutidas potenciais medidas de gestão que possibilitem mitigar os efeitos combinados das principais pressões estudadas tendo em conta os resultados obtidos.

Palavras-chave: Stresses-múltiplos, Mesocosmos, Biofilme, Macroinvertebrados, Escassez hídrica, Água residual

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SECTION I

FRAMEWORKING FRESHWATER MULTIPLE-STRESSORS

Chapter 1

General introduction and thesis outline

1. General introduction and thesis outline

1.1. Multiple-stressors in freshwater ecosystems

Aquatic ecosystems have been suffering drastic global alterations as consequence of their intensive use by human societies (Sala et al. 2000). Freshwaters, in particular, are among the most endangered ecosystems worldwide (Collen et al. 2014, Strayer and Dudgeon 2010), being threated and damaged by several pressures including geomorphological and hydrological modifications, changes in land use, over-exploitation of their natural resources, organic and inorganic pollution and invasive species (Ormerod et al. 2010, Vörösmarty et al. 2010). Additionally to these direct pressures, climate change represents a growing challenge to the integrity and functioning of freshwaters (Dudgeon et al. 2006). Such pressures can promote changes on water availability and also on ecological and chemical quality (Schinegger et al. 2012) leading to loss of habitat affecting biodiversity, biological integrity and ecosystem functioning, that consequently are prone to change the provision of key ecosystem services (Arce et al. 2014, Fausch et al. 2010).

Streams and rivers are particularly susceptible not only to diffuse and point source water pollution with a miscellaneous of organic compounds, pesticides, nutrients and pathogens released from urbanisation, industry and agricultural activities (Artigas et al. 2013**,** Heathwaite 2010, Perez et al. 2013), but also, have been heavily degraded with morphological alterations such as channelization, dams and deforestation, which together entail global menaces to human water security as well as river biodiversity (Vörösmarty et al. 2010).

An environmental pressure can generate multiple-stressors´ effects (e.g. urbanisation affects simultaneously runoff magnitude, water quality, temperature and habitat availability), but also several pressures of diverse sources can result in a same stressor (e.g. organic pollution, removal of riparian vegetation and water abstraction can all lead to water oxygen depletion). A stressor is defined as a measurable variable that exceeds its range of normal variation and affects (whether negatively or positively) individual taxa, community composition or ecosystem functioning in comparison to a reference condition (Crain et al. 2008, Piggott et al. 2015, Townsend et al. 2008). A stressor can be of natural or anthropogenic origin.

It is worth noting that the importance of multiple-stressors varies regionally (EEA 2012), e.g., in Alpine and upland northern Europe the freshwater systems are impacted mainly by hydropower plants effects, whereas in lowland areas of Europe and Mediterranean regions, freshwaters are impaired by intensive agriculture, riparian degradation, water scarcity and pollution.

The multitude of stressors acting in concert in freshwater systems have quite complex effects and often result in “ecological surprises” (Folt et al. 1999, Sheffer et al. 2001) that remain largely unknown, constituting a major pressing challenge for river basin managers and restoration ecologists (Hering et al. 2015). The fact is that, despite natural systems being most often under several simultaneous stressors, most ecological studies had been documenting stressors single effects on biota and ecosystems, while research into the cumulative and interactive impacts of multiple-stressors is less frequent. And despite the huge progress in the study of multiple stressors made more recently (Hearing et al. 2015, Jackson et al. 2016, Nõges et al. 2016), current river basin management practice seldom accounts for scientific evidence on the combined effects of multiple-stressors. Such lack of knowledge restricts the ability to understand ecosystem responses to multiple-stressors and recognize risk thresholds to define a hierarchy among stressors to classify priority actions, as their interactions are mostly unpredictable based on the knowledge of single stressors effects (Friberg et al. 2010, Hering et al. 2010, 2015, Ormerod et al. 2010).

The combined effect of diferent stressors can have three basic potencial outcomes classifed as i) additive (equal to the sum of the effects produced by the stressors acting in isolation), ii) synergistic (when the combined effect is larger than additive based on single stressors cumulative effect) or iii) antagonistic (when effects are smaller than the sum of isolated stressors) (Underwood 1989). In an antagonistic interaction, stressors may mitigate one another´s effects, while in a synergistic interaction, stressors amplify one another´s effect.

Most experimental studies addressing multiple-stressors employ treatments in a factorial design that include a Control, a stressor A, a stressor B and both stressors in combination (A + B) (Fig. 1.1). So, the interaction type (i.e. additive, synergistic or antagonistic) will vary depending on A + B response compared to the additive sum (AddS) of individual effects for stressor A and B in relation to Control (Fig. 1.1).

Recent studies have highlighted that stressors interactions in freshwater ecosystems may embody a large portion of all ecological responses (41% to 69%) (Jackson et al 2016, Schinegger et al. 2016) and that additive interactions may be as common as non-additive (i.e. synergistic and antagonistic interactions) (Nõges et al. 2016). Such observed differences may be dependent on the ecosystem type and the level of biological organization studied (from individuals to populations and ecosystems) (Côté et al. 2016). Thus, identifying the effect of every interaction on every organism´s physiology and every ecosystem function will be an impossible task given the rapid growing number of stressors and their potential interactions (Côté et al. 2016).

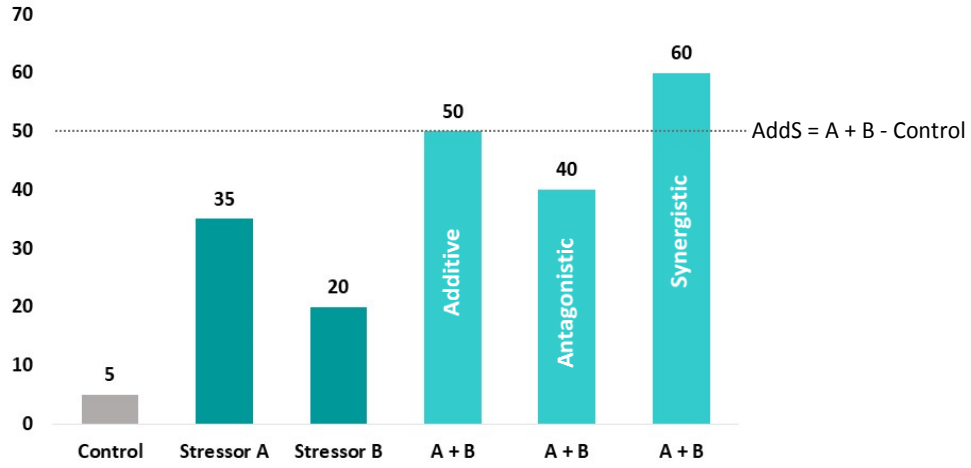

Figure 1.1. Conceptual categories of interaction types describing multiple stressors possible outcomes from data obtainable in studies with a factorial design. Interaction types are classified as Additive, Synergistic, and Antagonistic, depending on the A + B response compared to the additive sum (AddS) of individual effects for stressor A and B relative to the Control. Adapted from Crain et al. (2008).

1.2. Mediterranean ecosystems and their main challenges

Streams and rivers in semi-arid regions, such as Mediterranean basin, present a distinctive highly variable hydrological regime characterized by a flow intermittency, which are strongly linked to the seasonal temperature and rainfall patters of these regions (Gasith and Resh 1999). Accordingly, while during winter and spring periods Mediterranean rivers exhibit flood events, dependent on precipitation intensity and on morphology of the watershed (Fig.1.2A), during summer, rivers present very low flow with the often reduction of the wetted area to isolated pools or even to the complete dewatering of the river channel (Fig. 1.2B). This condition, associated with a high human water demand during summer, makes Mediterranean ecosystems largely vulnerable to water abstraction (Barceló and Sabater 2010, Gasith and Resh 1999).

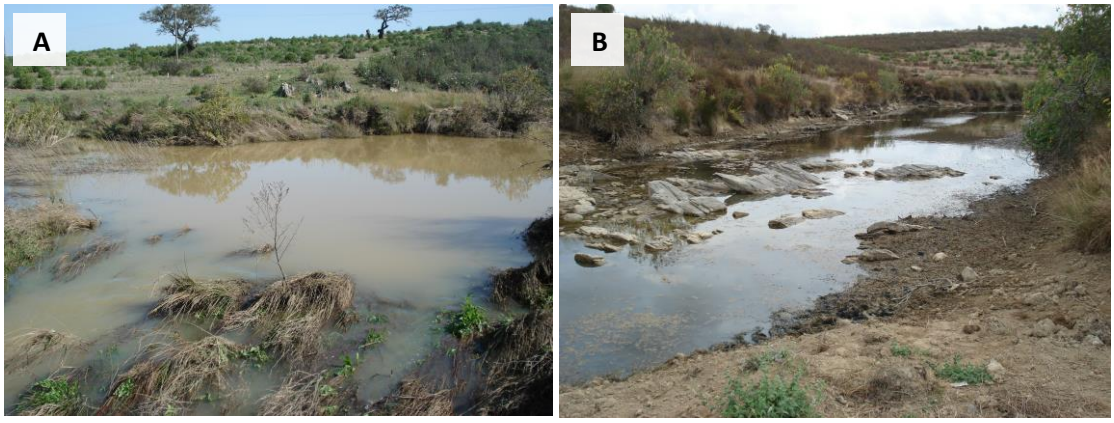

Figure 1.2. Mediterranean stream after a flood event (A) and during a drought period (B).

This type of systems can be found in regions of the world besides the areas surrounding the Mediterranean Sea, such as the southwestern South Africa, western and southern Australia, California and central Chile (Bonada and Resh 2013). Its climatic specificity (i.e. seasonality and inter-annual variability) has clearly a huge influence on communities and ecological processes. Consequently, the Mediterranean region has a high quantity of endemic species and is considered a global hotspot of biodiversity and related ecosystem services (Cuttelod et al. 2008, Myers et al. 2000). Nonetheless, it also contains the highest proportions of endangered freshwaters species. About one fifth (19%) of several species assessed in Mediterranean zones (either at regional or global level) are threatened by extinction and the main causes include habitat loss and degradation, pollution, overexploitation, natural disasters, invasive alien species and human disturbance (Cuttelod et al. 2008). In fact, Mediterranean streams and rivers have a long-lasting presence of human settlement and disturbance, which puts them among the most susceptible systems to environmental damage from human activities (e.g. water abstraction and diversion, river fragmentation, water contamination with organic and inorganic pollutants) (Dudgeon et al. 2006). Multiple-stressors action influence at least 31% of the water bodies under Mediterranean climate regions (Birk 2019).

Water scarcity is a major challenge presently faced by many societies, with particular emphasis in semi-arid regions such as the Mediterranean. Such pressure arises from the higher human water demand that exceeds the water resources exploitable under sustainable conditions (Barceló and Sabater 2010). Water abstraction for agriculture, industry and domestic use, the high number of dams and climate predictions of increased drought events are the major drivers to water scarcity within riverine ecosystems. For instance, Mediterranean European countries rely on rivers and streams to fulfil 64 to 100% of the water needs for irrigation (INAG 2001). Water use has been growing at more than twice the rate of population increases, while resources are decreasing. As consequence, water scarcity conditions prevail in most European Mediterranean countries particularly during summer, period in which water demands for agriculture, public supply and also touristic activities are even more pronounced (EEA 2018).
Even though flow decrease and intermittency are natural hydrological characteristics of Mediterranean rivers (Gasith and Resh 1999), water scarcity pressure may enhance its frequency and duration, also contributing to lentification of running waters, i.e. hydrological modification from lotic to standing waters (Sabater 2018). These may promote higher water temperatures and greater evaporation losses, markedly affecting water quality (Petrovic et al. 2011, Ponsatí et al. 2016) with direct and indirect effects on biota and ecosystem processes. Moreover, future climate scenarios foresee an exacerbation of water scarcity (frequency and duration of

droughts) in Mediterranean regions (IPCC 2014, Verdonschot and van den Hoorn 2010), which will increase the interaction with other persisting pressures including changes in land use, declining biodiversity and increasing pollution (Cramer et al. 2018, García-Ruiz et al. 2011).
By leading to intermittency of water flow with marked implications for hydrological connectivity, water scarcity can amplify the effects of other interacting stressors. Thus, the co-occurrence of a wide range of chemical, geomorphological and biological stressors under water scarcity may produce new and unknown interactions and potential strong effects with uncertain consequences.

As Mediterranean watersheds are densely populated, and thus, largely susceptible to anthropogenic pollution with water contamination from diffuse and point sources (Paul and Meyer 2001), the reduction of water quantity, under water scarcity conditions, will further favour water quality deterioration by reducing the natural dilution capacity of riverine ecosystems, while organic and inorganic pollutants concentrations increase (Petrovic et al. 2011, Ponsatí et al. 2016). The higher concentration of water pollutant substances will be concomitant with increases in water temperature, suspended solids, nutrient concentrations, conductivity, hypoxia and toxicants among other stressors (Barceló and Sabater 2010, Pardo and Garcia 2016). Particularly in these systems, sewage contamination and discharges from Wastewater Treatment Plants (WWTPs) can embody a large portion of the total stream discharge (Carey and Migliaccio 2009). In fact, domestic sewage effluents account for one of the most common causes of deterioration of water quality in streams (Paul and Meyer 2001) and despite most countries being investing in WWTPs to reduce that impact (Petrovic et al. 2011), they still represent a major point-source pollution in several riverine ecosystems (Carey and Migliaccio 2009). WWTPs outfall can input large amounts of organic matter and nutrients into receiving waters. Despite all the efforts for a better water cleaning with the employment of improved WWTP technologies (e.g. Tchobanoglous et al. 2003) resulting in significant reductions in chemical and organic pollutants, treatments are not able to remove all contaminants from wastewaters and consequently, the water discharge from WWTPs do not have the same quality as that of the receiving waters (Carey and Migliaccio 2009, Wakelin et al. 2008). Such effluents comprise a complex mixture of high organic loads and elevated levels of nutrients such as phosphorus, nitrogen, ammonium and nitrates (Paul and Meyer 2001) but also several other pollutants such as metals, pharmaceuticals and pesticides (Gibson and Meyer 2007, Huerta et al. 2016). Some of these substances such as pesticides and pharmaceuticals are toxic and have deleterious effects on stream biota and their activity (Corcoll et al. 2015, Ricart et al. 2009), while others such as nutrients can promote biological activity to a certain limit, beyond which an increased

in concentration also becomes stressful (Martí et al. 2001). In fact, an excessive concentration of organic compounds, nutrients and other pollutants derived from WWTs can be toxic at several levels of stream communities, from microbes to macroinvertebrates and fish (Arce et al. 2014, Baldy et al. 2007, Branco et al. 2016, Piggott et al. 2012). Such effects can alter consumption rates of key consumers, physiological condition and death rates that ultimately may affect ecological processes (e.g. organic matter decomposition, nutrient uptake efficiency; Bundschuh et al. 2013, Haggard et al. 2001, Feio et al. 2010), and the general trophic web (Bundschuh et al. 2011).

Overall, water scarcity and pollutants contamination are two of the most significant stressors acting in increasingly human-impacted Mediterranean rivers (Arenas-Sánchez et al. 2016, Petrovic et al. 2011), which produce complex effects that need to be addressed and quantified to help establish good management practices and effective restauration measures within these ecosystems.

1.3. Evaluating stressors with bioindicators: from primary producers to consumers

The Water Framework Directive (WFD; European Commission 2000) established the measurement of inland water quality by using several biological communities as quality indicators. Nevertheless, despite ecological assessment contemplating the determination of several pressures (e.g. pollution, habitat degradation and climate change) influence on quality elements, it neglects the need for consistent indicators that can discriminate between multiple-stressors effects (Hering et al. 2010).

Given that biological communities in river ecosystems are able to respond to disturbances faster than those in most other aquatic systems, biomonitoring has become a resourceful tool for assessing ecosystem health further than physical and chemical analyses (Moss 2007). Although a part of water quality can be assessed using just physicochemical measurements, it is mandatory in the European Union that member states evaluate ecological condition using key biological indicators such as fish, benthic invertebrates and primary producers like diatoms and macrophytes (Hering et al. 2006). Biological indicators respond to environment physical and chemical pressures with changes in composition, diversity and functions in the ecosystem, reflecting an overall ecological quality status (Friberg et al. 2010, White et al. 1998). These bioindicators incorporate the effect of diverse stressors over time and thus provide a measure of their combined impact trough temporal and spatial environmental fluctuations.

Benthic macroinvertebrates have been extensively used to evaluate ecological status and detect river impairments (e.g. Boix et al. 2010, Feio et al. 2015, Matthaei et al. 2010). They are a diverse and abundant group in most streams, with limited migration patterns or sessile nature, which constitutes good indicators for assessing site-specific impacts (upstream-downstream). They comprise species with a wide range of tropics levels, environmental tolerances and preferences and can provide strong information for interpreting cumulative effects (Buendia et al. 2013). Moreover, they represent important elements in freshwater ecosystems, contributing to their functioning through organic matter processing and energy transfer to consumers at higher trophic levels, among other processes (Heino 2005).

Besides biomass, taxonomic composition and behaviour, the use of species traits (e.g. mode of life, feeding habits, type of dispersal), has been helpful in discriminating overall human impacts (e.g. Buendia et al. 2013, Serra et al. 2017, Statzner and Beche 2010), because of traits spatial and temporal robustness and the systematic link they provide to main environmental drivers.

Overall, the high diversity of taxa and their potential responses to changes is a clear requirement for assessing effects of multiple stressors in lotic ecosystems, independently of the chosen bioindicator assemblage. Though, an approach based in just one specie seems limited towards the entire ecosystem responses, and a broad knowledge of biota interactions (e.g. biofilm microbial community) may help give insights into multiple-stresses impairments in the ecosystem.

Stream biofilms (Fig. 1.3.) are a very complex assemblage comprising a great microbial diversity as well as an elaborate 3-dimensional structure (Battin et al. 2003). This microbial layer that covers all surfaces in streams (e.g. stones, woody debris, sediment particles, plants) are composed of bacteria, archaea, protozoa, fungi, filamentous and single cell algae (e.g. diatoms) and even virus and other microfauna, which are embedded within a self-produced matrix of extracellular polymeric substances (EPS). This gelatinous matrix is composed mainly by water (up to 97%), but also includes extracellular polysaccharides, absorbed nutrients and metabolites, products from cell lysis (e.g. enzymes, DNA and RNA) and particulate material and detritus (Sutherland 2001). The biofilm matrix is a dynamic environment with microheterogeneity, capable of protect the cells within it (e.g. against river flow abrasion, predation) and enables chemical and physical communication among microorganisms though signals pathways.

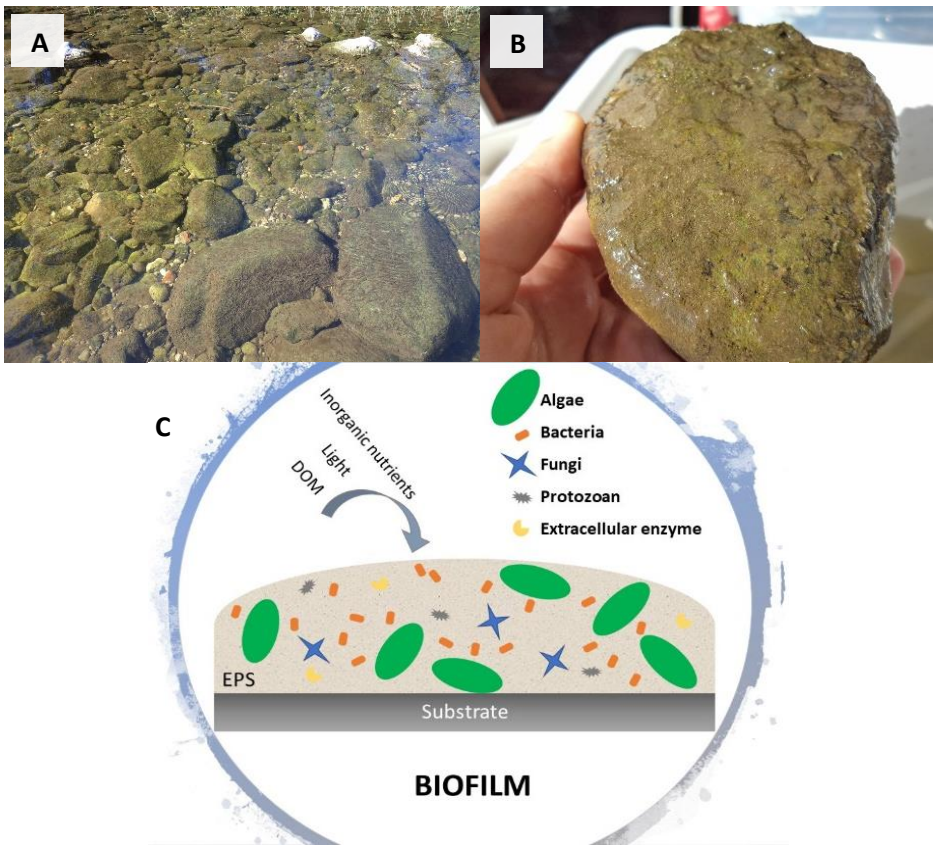

Figure 1.3. Stream biofilm (A, B). An idealized scheme of stream biofilm (C; based on Sabater et al. 2016), showing the different biofilm components (algae, bacteria and fungi as principals) embedded in an extracellular polymeric substances matrix (EPS), which are the receptors of inorganic and organic elements such dissolved organic matter (DOM) and are subjected to the effect of several environmental factors.

Stream biofilms play an important ecological role in freshwater ecosystems. As a whole, biofilm diverse microbial inhabitants act as a microecosystem where key ecological processes take place, such as primary production, carbon and nitrogen fixation and nutrient cycling. Major photosynthesis processes, energy and biomass production is carried out by algae and cyanobacteria, while biofilm-associated fungi, bacteria and protozoa contribute significantly to organic matter processing (e.g. decomposition of leaf litter in streams) and play important roles in carbon cycling and mineralization of nutrients in microbial food webs. Additionally, biofilms are a nutritional food resource for a range of macroinvertebrates and fishes. The feeding interactions of diverse heterotrophic organisms provide an important pathway for transferring nutrients and energy to higher trophic levels. The relative composition of microbial species in the biofilm potentially can influence their quality as food resource, thus affecting consumers' diversity, abundance and distribution patters in the ecosystem.

As river biofilms incorporate species with different roles and functions that rapidly respond to environmental perturbation, they are a good candidate to assess the impact of multiple stressors on rivers and streams (Sabater et al. 2007).

1.4. Research motivation

As previously mentioned, the fact that in most cases within freshwaters ecosystems multiple stressors act simultaneously in confounding and unexpected ways, and even though freshwater science and management are constantly dealing with such problem, only few studies actually provide quantitative evidence on multiple-stressors effects (Nõges et al. 2016), since there are not enough studies designed to understand their effects. Such breakthrough is determinant to have a better understanding of multiple-stressors main effects and mechanistic pathways, in order to help managers to define priority actions.

Besides, a better understanding of the effects of single pressures, multiple pressures and their interactions upon ecological status and ecosystem services is a clear prerequisite for effective river restoration (Palmer et al. 2005, Wohl et al. 2005) as well as proper watershed management.

Part of this Thesis (i.e. Section II) was developed under the scope of an EU-funded project – the MARS Project (Managing Aquatic ecosystems and water Resources under multiple Stress). One of MARS targets was to characterise relationships between multiple stressors and ecological responses, functions, services and water resources, and assess the effects of future land use and mitigation scenarios by working in 16 european river basins chosen to represent a wide range of multiple stress conditions, such as water scarcity and flow alterations in Southern Europe (addressed here), hydrology, morphology and nutrient alterations in Central Europe, and hydrology and temperature alterations in Northern Europe.

1.5. Main objectives and detailed aims

Knowledge on combined stressors effects and the relative importance of each stressor and their impacts on stream ecosystem structure and functioning is fundamental to support effective management and restoration measures. Both structural and functional changes in riverine biota are expected under the effect of multiple-stressors, in particular, water flow decreasing and sewage contamination. Therefore, the overall objective of this dissertation is to breakthrough on the knowledge regarding the response of different stream key biological communities to multiple stressors in Mediterranean rivers. It can be divided in 3 main parts composed by the experimental works. In the first part (corresponding to Section II) the main goal is to assess macroinvertebrate behavioural (i.e. drift) and functional (i.e. traits) responses towards the

effects of water scarcity (physical stressor) and oxygen depletion conditions (chemical stressor; as an outcome of organic loads). More specifically it aims to:

(1) evaluate macroinvertebrate drift differences with single and combined effects of flow reduction and hypoxia conditions,
(2) assess seasonal changes between stressors behavioural effects,
(3) investigate taxon-specific responses to the combined stressors,
(4) determine the changes in macroinvertebrate biological trait composition after exposure to stressors,
(5) evaluate which traits reveal better the single and combined effect of the stressors.

The second part (Section III) aims to investigate how single and combined effects of water flow stagnation and sewage contamination affect primary producers (i.e. stream biofilms) and primary consumers (i.e. grazers macroinvertebrates), by specifically aiming to:

(6) assess biofilm responses and investigate if stressors effects are up-scaled to the following trophic level through direct effects on grazers and indirect effects over biofilm quality as food resource,
(7) assess how biofilm microbial communities' proportions will change under the different stressors' combinations including also a natural stressor (i.e., grazing activity), by assessing biofilm associated bacteria, fungi and algae relative abundances through a molecular approach.

In the third part (Section IV), the main goal is to investigate multiple-stressors in a natural situation assessing the effect of flow and water quality variations in macroinvertebrate communities and in ecological condition by following a Mediterranean urban stream over time. Specifically, the aims were:

(8) analyse the effect of flow fluctuation and water quality variations in urban macroinvertebrate communities and in biological quality,
(9) test the use of specific pervasive taxa of urban streams identified at higher levels of taxonomic resolution and respective biological traits, as a finer tool to detect changes in the quality of urban streams under multiple-stressors.

1.6. Methodological approaches

Because anthropogenic stressors usually operate in concert and their interacting effects are very complex and difficult to unravel and predict (as mentioned before), is crucial to develop effective

methodologies and suitable experiments promoting the understanding of how multiple stressors interact and operate in freshwater ecosystems. Nevertheless, such studies are rare in streams, probably because of all the challenges involved in manipulating more than one stressor in a realistic experimental setting. Manipulative experiments allow for a controlled treatment of selected stressor levels to detect and quantify their effects on specific receptor metrics. The use of mesocosm experiments using factorial designs have been proven to be a better approach to deal with multiple-stressors in diverse contexts (e.g. Branco et al. 2016, Corcoll et al. 2015, Elbrecht et al. 2016, Matthaei et al. 2010, Piggott et al. 2012).

Therefore, the use of a mesocosm approach was chosen in most of the studies that compose this thesis (Fig. 1.4), in which the main objective was grounded on the assessment of single and interacting stressors effects in a factorial design.

Figure 1.4. Different mesocosms systems used in the experimental work of this thesis. A – Outdoor mesocosm. B – Indoor mesocosm set in acclimatized conditions of temperature and photoperiod.

1.7. Dissertation outline and organization

This thesis is composed by seven Chapters that are organized in five main Sections.

Section I – *Frameworking Freshwater Multiple-stressors* – consists in the Thesis´ introduction including Chapter 1, with the framework of the main theme, development of important concepts

within multiple-stressors problematic which are at the basis of the following chapters, and description of the main goals and specific objectives of the dissertation. The outline and organization of the thesis is also described within this chapter.

Section II – *Effects on Macroinvertebrate Responses* – incorporates Chapter 2 and Chapter 3, which reveal multiple-stressors effects on macroinvertebrate different responses tested under a controlled environment using an outdoor mesocosm system (artificial flumes).

Section III – *Responses of Stream Biofilms and Consumers* – includes Chapter 4 and Chapter 5, which demonstrates the multiple-stressors effects at different biota compartments, on primary producers and primary consumers, in experimental works performed in another type of mesocosm system (indoor artificial channels in an acclimatized room).

Section IV – *Multiple-stressors in the "Real World"* – consists in Chapter 6, illustrating a field study that deals with the assessment of biological responses to multiple-stressors in a natural situation (i.e. uncontrolled environment) by monitoring and sampling an urban stream *in situ*.

Lastly, **Section V – *General Discussion*** – includes Chapter 7 that is an overall discussion containing an overview of all experimental works presented in the previous chapters, taking into consideration the main objectives with main conclusions, final remarks and further research possibilities on freshwater biota under multiple-stressors.

Each of Chapters 2, 3, 4, 5 and 6 contained in Sections II, III and IV, correspond to experimental works that describe a stand-alone scientific manuscript that has been published, accepted for publication or submitted to a peer-reviewed journal. These chapters presentation corresponds essentially to the published or submitted version to the scientific journals, with some editing changes to ensure thesis´ format uniformization and improve readability.

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SECTION II

EFFECTS ON MACROINVERTEBRATE RESPONSES

Chapter 2

Macroinvertebrate short-term responses to flow variation and oxygen depletion: a mesocosm approach

Research article published in *Science of the Total Environment*

Calapez AR, Branco P, Santos JM, Ferreira TM, Hein T, Brito AG, Feio MJ (2017). Macroinvertebrate short-term responses to flow variation and oxygen depletion: a mesocosm approach. Sci Total Environ 599-600, 1202–121, DOI: 10.1016/j.scitotenv.2017.05.056

2. Macroinvertebrate short-term responses to flow variation and oxygen depletion: a mesocosm approach

Abstract

In Mediterranean rivers, water scarcity is a key stressor with direct and indirect effects on other stressors, such as water quality decline and inherent oxygen depletion associated with pollutants inputs. Yet, predicting the responses of macroinvertebrates to these stressors' combination is quite challenging due to the reduced available information, especially if biotic and abiotic seasonal variations are taken under consideration. This study focused on the response of macroinvertebrates by drift to single and combined effects of water scarcity and dissolved oxygen (DO) depletion over two seasons (winter and spring). A factorial design of two flow velocity levels – regular and low (vL) – with three levels of oxygen depletion – normoxia, medium depletion (dM) and higher depletion (dH) – was carried out in a 5-artificial channels system, in short-term experiments. Results showed that both stressors individually and together had a significant effect on macroinvertebrate drift ratio for both seasons. Single stressor effects showed that macroinvertebrate drift decreased with flow velocity reduction and increased with DO depletion, in both winter and spring experiments. Despite single stressors opposing effects in drift ratio, combined stressors interaction (vL×dM and vL×dH) induced a positive synergistic drift effect for both seasons, but only in winter the drift ratio was different between the levels of DO depletion. Stressors interaction in winter seemed to intensify drift response when reached lower oxygen saturation. Also, drift patterns were different between seasons for all treatments, which may depend on individual's life stage and seasonal behaviour. Water scarcity seems to exacerbate the oxygen depletion conditions resulting into a greater drifting of invertebrates. The potential effects of oxygen depletion should be evaluated when addressing the impacts of water scarcity on river ecosystems, since flow reductions will likely contribute to a higher oxygen deficit, particularly in Mediterranean rivers.

Keywords: Water scarcity, Hypoxia, Multiple-stressors, Synergistic effects, Drift, Mediterranean streams

2.1. Introduction

Aquatic ecosystems worldwide are exposed to complex mixtures of stressors mainly arising from anthropogenic sources such as overexploitation, water pollution, flow modification, destruction or degradation of habitat and invasion by exotic species (Dudgeon et al. 2006, Fausch et al. 2010, Schinegger et al. 2012). In European running waters, such factors seldom operate in isolation (Schinegger et al. 2016), and their impacts on stream ecosystems generally reflect a combined response to multiple stressors, generating changes on water availability and on ecological and chemical quality with complex entangled effects on biodiversity, ecological processes and ecosystem functioning and management (Brown et al. 2013, Ormerod et al. 2010, Townsend et al. 2008). Consequently, multiple-stressors responses are often unpredictable based on the knowledge of single stressors and attempts to understand "ecological surprises" that may outcome from interacting stressors are a pressing challenge (Friberg 2010, Hering et al. 2010, Hering et al. 2015, Ormerod et al. 2010). These complex responses can be synergistic or antagonistic, when the combined effect is respectively larger or smaller than additive (when the response is given by the sum of the effects produced by the stressors acting in isolation) (Underwood 1989).

Freshwater systems under Mediterranean climate are particularly influenced by severe hydrological fluctuations related with their natural intermittent flow regime (Gasith and Resh 1999) but also strongly associated with direct and indirect anthropogenic factors such as water abstraction, land use and ultimately climate change (Bonada and Resh 2013, Ludwig et al. 2011). In fact, future climate change scenarios predict an exacerbation of water scarcity (frequency and duration of droughts) in Mediterranean regions (IPCC 2014, Verdonschot and van den Hoorn 2010), which will increase the interaction with other anthropogenic stressors. Water pollution from diffuse and point sources is a major threat to rivers within this climatic zone (Manfrin et al. 2013, Ortiz et al. 2005, Perujo et al. 2016). The strong effects of domestic and industrial sewage effluents on these ecosystems are intensified under reduced flow conditions and slower current velocities since dilution capacity is largely diminished (Prenda and Gallardo-Mayenco 1996). Furthermore, decreases in water availability are frequently associated to increased water temperatures, high nutrient and suspended solid concentrations, and decreases on dissolved oxygen concentrations (DO) (Miller et al. 2007, Stanley et al. 1997). Indeed, water scarcity and DO depletion are two of the most significant stressors acting in increasingly human-impacted Mediterranean rivers (Arenas-Sánchez et al. 2016, Petrovic et al. 2011).

Although oxygen deficit conditions in aquatic ecosystems can occur under natural conditions (Acuña et al. 2005, Hladyz et al. 2011), oxygen depletion is more commonly the result of the

build-up of organic wastes and eutrophication (Díaz and Rosenberg 2011, Franklin 2014). As the inflow rate of organic pollutants in the water increases, the concentration of DO decreases. Small Mediterranean streams are greatly impaired not only by regular sewage inputs, but also by episodic inputs from small industrial plants or domestic sources (Manfrin et al. 2013). Even though hypoxia conditions caused by episodic sewage inputs disappear shortly downstream from the discharge point, local impact on stream communities may be particularly critical when flow is limited (Pardo and García 2016).

Benthic macroinvertebrates are a key biological element within lotic systems, widely used to evaluate ecological status and detect river impairments (e.g. Boix et al. 2010, Feio et al. 2015, Matthaei et al. 2010). They comprise species with a wide range of trophic levels, environmental tolerances and preferences, and can provide strong information for interpreting interaction effects (Rosenberg and Resh 1993). Macroinvertebrate drift rates can respond to short term fluctuations in environmental impacts. Drift mechanism can be a consequence of passive (e.g. accidental dislodgment) or active drift (e.g. avoidance behaviour) and enables the organisms to escape unfavourable conditions, giving them the potential to colonize new habitats.

An overall decreasing in macroinvertebrate density and an increasing in downstream drift have been reported as usual responses of lotic macroinvertebrates to several types of disturbance including water pollution (Feio et al. 2010, Friberg 2010, Ortiz et al. 2005) and inherent hypoxia (Rueda et al. 2002), and alterations in flow regime (Gibbins et al. 2010, Schülting et al. 2016). Low flow periods often result in an increase in limnophilic taxa to the detriment of rheophilic (Jowett and Duncan 1990). While drift rate has manifold responses towards slow current velocities, translating into a lower (Dewson et al. 2007), higher (Acuña et al. 2005) or no change (James et al. 2008) abundance/density in local taxa, water DO depletion have been consistently found as a deleterious factor in water dwelling organisms (Burnett and Stickle 2001, Cox 2003, Pardo and García 2016). Additionally, community composition and abundance are expected to change between seasons (Hieber et al. 2003, Stoneburner 1979), so understanding these stressor effects should take into account seasonal variations of communities' sensitivities and vulnerabilities, since they can change temporally during the lifespan of an organism and also differ between life stages (Segner et al. 2014). On the other hand, the seasonal aspect also acts directly through changes in abiotic elements such as water temperature and flow magnitude, which also have an effect on macroinvertebrates (Miller et al. 2007). Therefore, the quantification of stressors single and combined effects and identification of biota response mechanisms to these impacts are difficult to study *in situ*, where several uncontrollable stressors are at play (Barceló and Sabater 2010, Ormerod et al. 2010). As a result, mesocosm systems have been extensively used in stream ecosystem research to improve understanding of multiple

stressor effects on freshwater biota (e.g. Branco et al. 2016, Elbrecht et al. 2016, Jones et al. 2015, Matthaei et al. 2010, Piggott et al. 2012, Poff et al. 2003, Schülting et al. 2016). Yet, to our knowledge, macroinvertebrate instantaneous drift patterns still remain poorly understood in the context of interacting effects of reduced flow and increasing oxygen deficit under different seasons.

In this study, an experimental stream setup with artificial channels was used to assess macroinvertebrate drift towards single and combined effects of water scarcity (physical stressor) and oxygen depletion conditions (chemical stressor) over two sequential seasons (winter 2015 and spring 2016). Our goals are: (1) to evaluate macroinvertebrate drift differences with single and combined effects of flow reduction and oxygen depletion; (2) to assess seasonal changes between stressors effects; and (3) to investigate taxon-specific drift responses to the combined stressors.

Overall, we expect that low flow velocity effect on community drift will depend on taxon preference for slower or higher flow velocity. Also, drift response will be enlarged by DO depletion effect, and this effect will be more drastic with a higher level of DO deficit in the water column. We hypothesize that the combination of low flow velocity with oxygen deficit conditions (even though for a short term) will have an amplified effect and will increase macroinvertebrate drift response. Lastly, we predict that macroinvertebrate drift response magnitude to the single and combined stressors effects will be dependent on season, although both possibilities could happen: in spring the higher water temperature might worsen hypoxia effects leading to a higher drift; but, in winter most macroinvertebrate taxa should be in an earlier successional stage of life cycle and therefore might be more vulnerable to the stressors, resulting also in a higher drift behaviour.

2.2. Material and methods

2.2.1 Experimental facility and setup

The individual and combined effects of flow reduction and increasing DO depletion pulses were tested in winter (December 2015 – January 2016) and spring (May – June 2016) at a mesocosm system located in the campus of School of Agriculture in Lisbon, Portugal. The mesocosm design (Fig. 2.1) consisted in a set of outdoor artificial flumes composed by 5 independent stainless-steel-lined channels (width 0.4 m × length 4 m × depth 0.2 m). Water was supplied from an *in situ* natural spring (pH = 8.06; Conductivity = 865 µS/cm; DO = 9 mg/l) to a central tank (3000 L),

and then distributed to head containers (70 L) in each channel evenly. Each flume runs to a downstream tank (70 L) connected to a pump (Kripsol OK-71 B, 0.56 kW) operating in recirculation flow system to the head container which allowed upholding water conditions independently of the source tank. Water distribution and recirculation was ensured with a PEAD pipe system. Fresh water was supplied to the central tank for each treatment and then used in the entire system during the experiment.

The experimental area (0.4 m x 0.6 m = 0.24 m^2) was set at the lowermost section of each flume. This section was covered with pebbles to mimic natural stream bed and was delimited upstream with a mesh framed panel. At the outflow of each flume, a removable drift net (500 µm mesh size) was placed to collect drifted taxa.

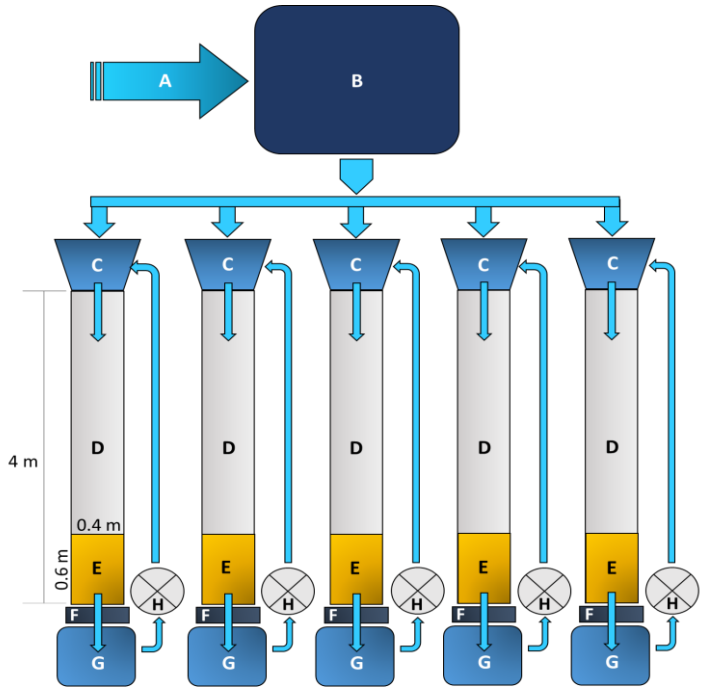

Figure 2.1. Schematic overview of experimental system setup. Water was recirculating in each flume. A – water source inlet from a spring, B – central source tank with distributing pipe system, C – head containers, D – flume, E – experimental area, F – drift net, G – downstream tank, H – pump.

Macroinvertebrate test population was collected at a sample site in River Jamor (Tagus basin, central Portugal), a small (16 km long) coastal Mediterranean river. Located 8 km west of the mesocosm facility, River Jamor drains a 44.5 km^2 catchment, whose uppermost reaches are forested, but otherwise alternates between urban areas and remnant agricultural and open-space lands. The lithology is dominated by sedimentary rocks (limestone and sandstone). Site selection was based on habitat representativeness, i.e., taking into account the presence of well-defined run-riffle sections and lack of severe human impacts (pH = 8.8 ± 0.6; Cond = 674.2 µS/cm ± 50.3; DO = 10.7 mg/l ± 1.0; Mean ± SD). A total of 30 samples per season were taken with a

hand net (500 µm mesh size, 25 × 25 cm frame size) by kick sampling. Each sample, corresponding to an area of 0.24 m^2, was composed by four subsamples: two samples taken in a run habitat near the bankside (with the lower flow velocity) and two samples taken in a riffle habitat (with the higher flow velocity) to ensure a diverse community in terms of flow velocity preferences. Macroinvertebrate samples were transported in a cooler to laboratory and then acclimatized with the water of the mesocosm system for at least 24 h before being transferred to each flume. Afterwards they were acclimatized to channel conditions for at least 1 hour *prior* to the test, maintaining a water column of approximately 8 cm in depth. This process was repeated for each treatment replication, i.e. 5 fresh macroinvertebrate samples were tested for each experiment (n = 6) in successive days.

2.2.2 Experimental design and procedure

To assess the drift response to flow reduction and increasing DO depletion pulses, we manipulated 2 flow velocity levels [Regular (vC) = 0.2 ± 0.03 m/s; Low (vL) = 0.06 ± 0.03 m/s] and 3 levels of oxygenation conditions [Normoxia (dC) = no DO reduction; medium DO depletion (dM) = 1 pulse of DO reduction per hour; higher DO depletion (dH) = 2 pulses of DO reduction per hour] during three hours with five synchronous replicates. This factorial design, resulted in six experimental treatment combinations testing the single and combined effects of the stressors: (1) Regular flow with normoxia (vC+dC) as control treatment; (2) Low flow with normoxia (vL+dC) as single effect of water scarcity; (3) Regular flow with medium DO depletion (vC+dM) as single effect of medium DO depletion; (4) Regular flow with higher DO depletion (vC+dH) as single effect of higher DO depletion (5) Low flow with medium DO depletion (vL+dM) as combined effect of water scarcity and medium DO depletion; (6) Low flow with higher DO depletion (vL+dH) as combined effect of water scarcity and higher DO depletion.

Since Mediterranean streams have characteristically a wide variation of flow velocity levels (Gasith and Resh 1999), in this study a regular flow velocity was defined as control, corresponding to the medium flow velocity observed within this climatic region outside flood and dry seasons, and a lower velocity which can be observed frequently when the system starts to contract in the dry season. Lowering the water flow-velocity induced also a decreasing in the water depth *in* channel (ca. 1 cm). Higher level of oxygen depletion was chosen to be near the threshold that starts to cause stressful conditions to some aquatic organism (4 mg/L; ca. 45% DO) (Franklin et al. 2014) and induce a strong drift response in moderate to tolerant macroinvertebrate species, whereas a medium oxygen depletion (ca. 60% DO) was selected as the saturation in which most sensitive species actively enter the drift (Connolly et al. 2004). The

pulses of DO depletion were induced through the addition of sodium sulphite (Na_2SO_3) (Park et al. 2014) into the water of each flume, by dissolving an initial amount of 50 g for the first DO reduction pulse for both DO depletion treatments. This baseline DO reduction was followed by an addition of 25 g of Na_2SO_3 for the subsequent pulses according the frequency established for each treatment (i.e., 1 pulse per hour for dM and 2 pulses per hour for dH treatments). This compound is an acknowledged oxygen scavenger (Lewis 1970) that has been used in the setting of oxygen-deficit conditions in water research (Branco et al. 2016, Crampton 1998, Peay et al. 2006). Preliminary tests showed that these amounts of Na_2SO_3 were sufficient to reduce DO momentarily within a range of 30% to 60% DO (in relation to the control situation – vC+dC). This artificial oxygen depletion was used as a proxy for the decreasing of DO in rivers due to the degradation of organic matter and microbial processes inherent of point organic loads in the system. Flow velocity levels were measured for each test with a digital water velocity meter (model FP101, Global Water Instrumentation, USA). Temperature (°C), pH, conductivity (µS/cm), and total dissolved oxygen (%DO) were monitored in all flumes throughout the experiment (n = 4) for each treatment combination using portable meters (HANNA´s multiparameter probe HI 9812-5 and oxygen probe HI98193).

Drift net contents were collected after treatment exposure and preserved with ethanol (96%) for later macroinvertebrate sorting, counting and identification. Also, the remaining individuals in the flumes (no drift samples) were flushed out with flowing water to a drift net by carefully washing and stirring the sediments by hand. Drift and no drift macroinvertebrate samples were processed individually to infer the initial overall tested community. Between each experiment, all flumes were entirely washed with flowing water and the sediments were fully removed from the experimental area for further washing and drying before being used again.

Macroinvertebrate taxonomic identification, under a stereomicroscope (Leica M80), was done to the lowest possible taxonomic level (i.e., genus and species level), with the exception of Diptera (identified to family, subfamily or tribe level) and Oligochaeta (family).

Taxa information regarding functional and biological traits relate with the studied stressors such as respiration mode and current preferences, was attained according to the available physiological and ecological information databases described in Tachet et al. (2000) and freshwaterecology.info (Schmidt-Kloiber and Hering 2015).

2.2.4. Data analysis

The drift response was measured as the ratio of drifted individuals per total number of tested individuals (i.e. relative abundance of taxa that drifted) in order to enable comparisons between

treatments and also between seasons. Macroinvertebrate drift data consisted of relative abundances >0.1%, and taxa present in only one sample were not considered because rare taxa normally have a minor influence on results of multivariate statistics and can be seen as outliers in ordinations (Gauch 1982). Taxa drift ratio were fourth-root transformed to down-weight the influence of few abundant taxa prior to analysis. Significant differences ($p<0.05$) for macroinvertebrate community drift ratio for each stressor and their interaction were tested for winter (w) and for spring (s) using permutational multivariate analysis of variance (PERMANOVA: unrestricted permutations of raw data (9999 randomisations; Anderson 2001) for the two fixed factors "flow" and "oxygen" with two (vC and vL) and three levels (dC, dM and dH) respectively, based on a Bray-Curtis similarity matrix. Pair-wise post hoc tests were performed whenever an overall effect was significant.

The expected drift ratio if the two stressors had an additive effect (i.e. cumulative effect of single stressors without interaction) was determined. For that purpose, the predicted macroinvertebrate overall drift ratio (PDR) in a scenario of water scarcity and point DO depletion was calculated from the drift ratio in a control situation (vC+dC), by a decreased in flow velocity alone (vL+dC) or an increased in DO depletion alone (vC+dM and vC+dH) assuming no interaction between factors. The interaction type and directional classification for the two-stressor response was assessed based on the conceptual approach of Piggott *et al.* (2015a). This classification system is based on an additive effects model that combines the magnitude and the response direction (+ or −) of the cumulative effect (effect of combined stressors relative to control) and interaction effect (effect deviation from the additive model prediction), in order to determine synergism and antagonism relative to individual stressor effects in absolute terms. Directional interaction classes are *additive* (AD), *positive synergistic* (+S), *negative synergistic* (-S), *positive antagonistic* (+A) and *negative antagonistic* (-A) depending on the sum of both stressors' effects compared to the additive sum of individual stressors effects relative to the control. A difference from additive that is greater than the sum of individual effects and greater than any individual effect in the same direction or has an interaction effect that is greater than both in absolute terms is +S (more positive than predicted additively) or −S (more negative than predicted additively). An interaction that differs from additive and is less than the sum of the individual effects or less-than or-equal-to any individual effect is +A (less positive than predicted additively) or -A (less negative than predicted additively).

To assess the patterns of macroinvertebrate community drift response to treatments effects for both seasons, a non-metric multidimensional scaling (NMDS) (50 randomised starts) based on Bray-Curtis similarities was performed. Seasonal differences between stressors effects in macroinvertebrate drift ratio were also tested by performing unrestricted permutations of raw

data (9999 randomisations; PERMANOVA), including “season”, “oxygen” and “flow” velocity as fixed factors, with two levels (w and s) for season and the same levels as before for the remaining factors. Seasonal differences were tested based only in the common taxa between winter and spring (13 taxa) and therefore a total of 7 unshared taxa were excluded from these analyses.
All statistical tests were performed using PRIMER 6 & PERMANOVA+ software (PRIMER-E Lda, Plymouth UK) (Anderson 2001).

2.3. Results

2.3.1 Experimental conditions and macroinvertebrate samples

Manipulated factors in this study (flow velocity and oxygen level) for each treatment are shown in Figure 2.2. Mean flow velocity was reduced in approximately 65% from vC to vL treatments and upheld within the same range for both seasons (between 0.16 and 0.20 m/s for vC and between 0.05 and 0.07 m/s for vL). Mean water DO levels decreased about 33% for dM and 51% for dH and this pattern was verified for both studied seasons (Fig. 2.2) (i.e. mean DO decreased from dC = 100% ± 4 SD to dM = 67% ± 7 SD, and to dH = 49% ± 24 SD, for both seasons).
Parameters monitored during the experiment such as pH and water temperature remained rather constant among different treatments throughout the experiment for both seasons: mean pH was 9.1 ± 0.4 SD in winter and 8.4 ± 0.4 SD in spring, while mean water temperature was 16.3°C ± 1.3 SD in winter and 22.3°C ± 1.4 SD in spring (Table 2.1). However, it was registered an increase in conductivity mean values for both seasons, corresponding to the increase of DO depletion from dC to dM to dH treatments (Table 2.1).
For each treatment (n = 6), a total of 4534 ± 1127 (mean ± SD) and 6154 ± 1597 (mean ± SD) specimens were used to test the stressors effects during winter and during spring, respectively, comprising a total of 40 different taxa for each season. Ephemeroptera, namely *Baetis* sp., *Acentrella* sp. and *Caenis* sp. were the most common and abundant taxa in all initial samples used to test the stressors, representing up to 90% of the total individuals in experiments for both seasons. Other representative taxa included *Hydropsyche* sp. (Trichoptera) (4%) and Simuliini (Diptera) (3%) for winter samples and *Echinogammarus* sp. (Crustacea) (5%) and *Hydroptila* sp. (Trichoptera) (3%) for spring samples.

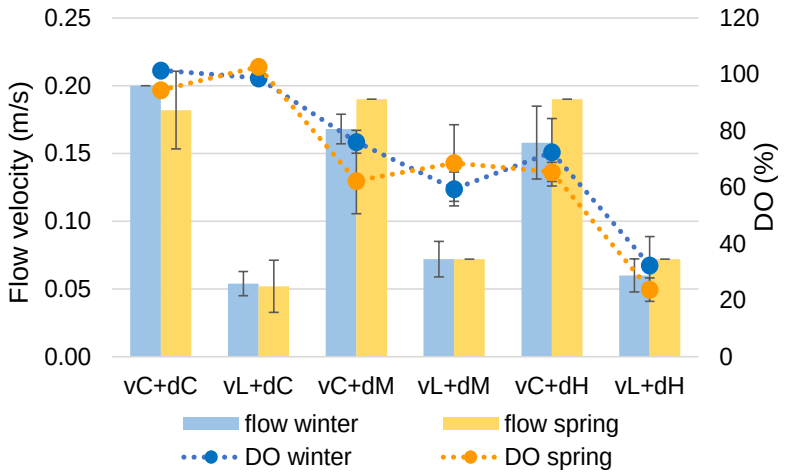

Figure 2.2. Mean flow velocity ± SD (bars; m/s) and mean dissolved oxygen concentration ± SE (lines, %) during the experiment for the different treatment combinations for winter (blue) and spring (orange). vC+dC – control, vL+dC – low flow velocity effect, vC+dM – medium DO depletion effect, vL+dM – combined effect of low flow and medium DO depletion, vC+dH – higher DO depletion effect, vL+dH – combined effect of low flow and higher DO depletion.

Table 2.1. Physical-chemical parameters (mean ± SD) measured during the experiment for each treatment combination during winter and spring. vC+dC – control, vL+dC – low flow velocity effect, vC+dM – medium DO depletion effect, vL+dM – combined effect of low flow and medium DO depletion, vC+dH – higher DO depletion effect, vL+dH – combined effect of low flow and higher DO depletion.

Treatment	Temperature (°C)				pH				Conductivity (µS/cm)			
	Winter		Spring		Winter		Spring		Winter		Spring	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
vC+dC	16.3	0.3	23.7	0.8	9.4	0.1	8.2	0.1	682	30.5	532	23.3
vL+dC	15.8	0.2	21.8	0.7	9.6	0.1	7.9	0.1	644	31.5	503	40.2
vC+dM	17.2	0.3	21.8	0.3	8.5	0.1	8.7	0.1	1082	64.6	1076	47.7
vL+dM	15.5	0.0	21.3	0.4	9.3	0.0	8.7	0.0	1307	101.5	1244	53.4
vC+dH	17.0	0.1	22.7	0.1	8.9	0.0	8.7	0.0	1454	127.1	1370	42.8
vL+dH	16.2	0.6	22.8	0.2	9.0	0.0	8.6	0.0	1614	72.3	1280	23.7
Mean	16.3		22.3		9.1		8.4		1130.5		1000.7	
SD	0.7		0.9		0.4		0.4		402.5		386.4	

2.3.2. Stressors single and combined effects on macroinvertebrate community drift – flow velocity and oxygen

Results given by PERMANOVA testing the effect of flow velocity and oxygen condition in each season (Table 2.2), showed that both factors and their interaction had a significant effect on macroinvertebrate community drift rate.

The single exposure to low flow velocity (vL), significantly reduced macroinvertebrate drift ratio for both winter and spring experiments (Table 2.2), when comparing to control (vC) (PERMANOVA, pair-wise tests; $t=2.1359$, $p<0.05$ for winter and $t=1.8861$, $p<0.05$ for spring). Concerning oxygen depletion single effects in winter, both medium DO depletion (dM) and higher DO depletion (dH) showed a significantly higher drift ratio when compared to normoxia (dC) (PERMANOVA pair-wise test; $t=2.0623$, $p<0.05$ and $t=2.5617$, $p<0.05$, respectively), and comparing both levels of oxygen depletion, dH treatment showed also a higher drift ratio than the dM treatment (PERMANOVA pair-wise test; $t=1.8502$, $p<0.05$). For spring, the same drift pattern was observed, i.e., there was a significant drift ratio increase from dC to dM to dH treatments [PERMANOVA pair-wise test; $t=1.6863$, $p<0.05$ (dC<dM); $t=2.546$, $p<0.05$ (dC<dH); $t=1.5978$, $p<0.05$ (dM<dH)].

Table 2.2. Effects of single and interacting factors on macroinvertebrate drift ratio for both seasons. (PERMANOVA, 9999 unrestricted permutations of raw data based on Bray-Curtis similarity matrix). Significant p-values ($p<0.05$) are in bold. Rankings based on absolute values for post hoc tests for factors levels combinations are also shown. vC – regular flow velocity, vL – low flow velocity, dC – normoxia, dM – medium DO depletion, dH – higher DO depletion.

Source of variance	**Winter**			**Spring**		
	Pseudo-*F*	p-value	Ranking pairwise test (significance p<0.05)	Pseudo-*F*	p-value	Ranking pairwise test (significance p<0.05)
Oxygen (Ox)	4.6781	**0.0001**	dC < dM < dH	3.9103	**0.0001**	dC < dM < dH
Flow (Fl)	7.8575	**0.0001**	vC > vL	3.911	**0.0001**	vC > vL
Ox × Fl	2.841	**0.0019**		1.8794	**0.0404**	
Fl stressor:						
vL			dC < dM < dH			dC < (dM = dH)
Ox stressor:						
dM			vC < vL			vC = vL
dH			vC < vL			vC < vL

A significant interaction of flow velocity and oxygen condition was detected in macroinvertebrate drift for both seasons using a PERMANOVA with two fixed factors (Table 2.2). PERMANOVA pair-wise comparisons for this interaction showed that in winter macroinvertebrate drift ratio within low flow velocity (vL), increased significantly with the decreasing of DO concentration in the water with a higher drift for dM ($t=1.8258$, $p<0.05$) and dH ($t=2.6918$, $p<0.05$) compared to dC and also an increase drift ratio from dM to dH ($t=1.9214$, $p<0.05$). For spring, within low flow velocity (vL), drift ratio increased significantly from normoxia treatments (dC) to oxygen depletion treatments, dM ($t=1.5688$, $p<0.05$) and dH ($t=2.3511$,

p<0.05) independently of the level of DO depletion frequency pulses tested (i.e., dM=dH) (Table 2.2). Also, within medium DO depletion (dM), drift ratio was higher under vL only in winter (t=2.2845, p<0.05), whereas for higher DO depletion (dH), drift was significantly higher for both seasons (t=1.9035, p<0.05 for winter and t=1.8205, p<0.05 for spring) (Table 2.2).

2.3.3. Interaction between water scarcity and oxygen depletion on total drift rates

Overall macroinvertebrate drift ratio (Fig. 2.3) measured for water scarcity and oxygen depletion treatments, followed the same pattern that was tested for community drift ratio (Table 2.2). Comparing the individual stressors effects (vL+dC, vC+dM and vC+dH) with the interaction effect from combined low flow and both levels of oxygen depletion treatments (vL+dM and vL+dH), is clear a trend for higher drift ratios with the interaction of stressors, for both seasons (Fig. 2.3).

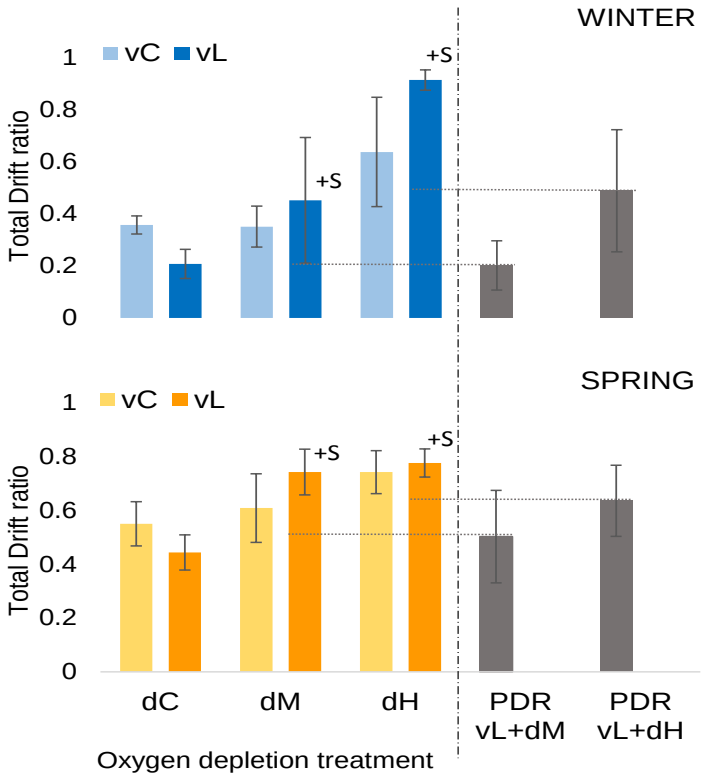

Figure 2.3. Macroinvertebrate total drift ratio (mean ± SD) for the three oxygen conditions (dC – normoxia, dM – medium DO depletion, dH – higher DO depletion) and the two flow velocity levels (vC – regular flow velocity, vL – low flow velocity) tested for both seasons. The grey bars indicate the predicted drift ratio (PDR) under combined stressors (vL+dM and vL+dH) given by the additive sum of individual effects relative to control. Directional interaction class between combined stressors are shown (positive synergistic; +S).

For both seasons, total drift response observed in our experiment for the interaction of low flow and oxygen depletion (both levels; vL+dM and vL+dH) was higher than the predicted (PDR vL+dM and PDR vL+dH; Fig. 2.3) showing a positive synergistic effect (+S) between factors, taking into

account the direction of individual stressors effects and interaction effect (*sensu* Piggott *et al*. 2015a). In detail, during winter, both low flow treatment (vL+dC) and medium DO depletion (vC+dM) had a negative effect, but had a double positive effect for the interaction type, i.e. the observed interaction effect (vL+dM) was more positive than what would be predicted by adding the effects of the single stressors (PDR vL+dM) and more positive than individual effects (Fig. 2.3, WINTER). With the higher DO depletion level, the direction of individual stressors was opposing (negative for vL+dC and positive for vC+dH), but the interaction effect was also double positive (Fig. 2.3, WINTER). For spring, the same positive synergistic effect (+S) was observed for both DO depletion levels tested. The observed interaction effect was more positive than predicted additively (Fig. 2.3, SPRING).

2.3.4. Seasonal influence of stressors effects on macroinvertebrate drift

Focusing on the commonest 13 taxa tested for both seasons, the NMDS (stress = 0.19) plot showed a clear segregation in macroinvertebrate drift response between winter and spring for all treatments (Fig. 2.4). Is also visible that spring drift responses were more similar to each other than in winter. This seasonal difference in macroinvertebrate drift ratio was confirmed with PERMANOVA main test result (Table 2.3), which showed a significant effect of the season in discriminating drift behaviour. Moreover, drift ratio was also influenced by the interaction Season × Oxygen and influenced by the interaction of season with both factors (Season × Oxygen × Flow) (Table 2.3).

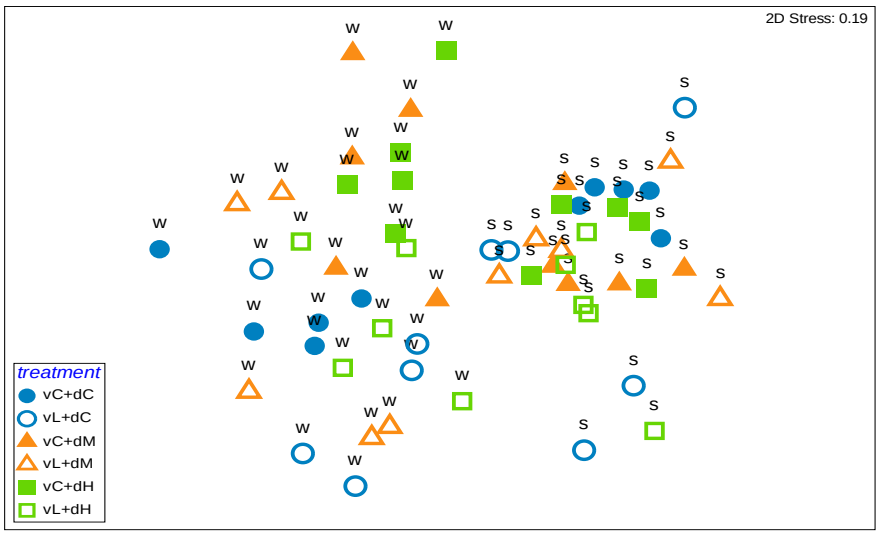

Figure 2.4. Macroinvertebrate drift response for 3 factors (oxygen level, flow velocity and season) determined by non-metric Multi-Dimensional Scaling (NMDS) ordination. Plot shows drift ratio across the two levels of flow velocity and the three levels of oxygen depletion for both seasons. Different letters specify seasons studied (w - winter, s- spring). Different colours indicate oxygen depletion levels (blue – control; orange – medium depletion; green – higher depletion) and open/filled symbols indicate flow velocity condition (filled - regular flow; open – low flow).

Table 2.3. Effects of Season and interaction with the stressors (Ox - oxygen; Fl - flow velocity) on the macroinvertebrate drift ratio. (PERMANOVA, 9999 unrestricted permutations of raw data based on Bray-Curtis similarity matrix). Significant p-values (p<0.05) are in bold.

Source of variance	Pseudo-*F*	p-value
Season (Se)	42.516	**0.0001**
Se × Ox	4.403	**0.0001**
Se × Fl	2.1913	0.059
Se × Ox × Fl	2.3601	**0.0182**

2.3.5. Taxon-specific drift seasonal patterns to single and combined stressors

Regarding overall specific taxon drift behaviour (Fig. 2.5), a general trend to a higher drift proportion in oxygen depletion conditions (dM and dH treatments) for both seasons was revealed, while decreased flow velocity seemed to lower drift response for almost every taxon. In winter experiments, taxa such as Ephemeroptera (*Acentrella* sp. and *Baetis* sp.), Diptera (Chironomini, Orthocladiinae and Tanipodinae), *Hydropsyche* sp. (Trichoptera) and *Proasellus* sp. (Crustacea), showed a particular lower drift response under flow velocity decrease (vL+dC) comparing with the control (vC+dC). However, under combined stressors, these taxa showed a higher drift ratio with low flow × oxygen depletion effect (both tested levels) comparing with low flow single effect.

In the spring experiment, Simuliini (Diptera) displayed an evident higher drift ratio effect to low flow velocity (vL+dC) comparing to the remaining treatments. Contrary, taxa such as *Caenis* sp., *Elmis* sp., *Proasellus* sp. and Tanipodinae decreased their drift response with low flow treatments (Fig. 2.5). But, when combining both stressors (low flow × DO depletion) these same taxa showed a significant increase in their drift ratio response. *Echinogammarus* sp. specimens showed a particular strong drift reaction (about 62% drift rate) to the combination of low flow and the higher DO depletion level (vL+dH), when comparing to the remaining treatments (Fig. 2.5).

Different trends in drift ratio proportions between seasons were observed (Fig. 2.5) in taxa such as Baetidae (*Baetis* sp. and *Acentrella* sp.), Chironomini, Tanytarsini, Simuliini, *Hydropsyche* sp. and *Proasellus* sp.. Individuals of the Diptera tribe Tanytarsini did not drift in any treatment during winter, and in spring presented a lower drift proportion in vL treatments regardless of DO condition. Despite Orthocladiinae specimens not responding to any treatment during spring experiments, in winter these individuals presented a lower drift response in vL treatments when compared to vC treatments, independently of DO conditions.

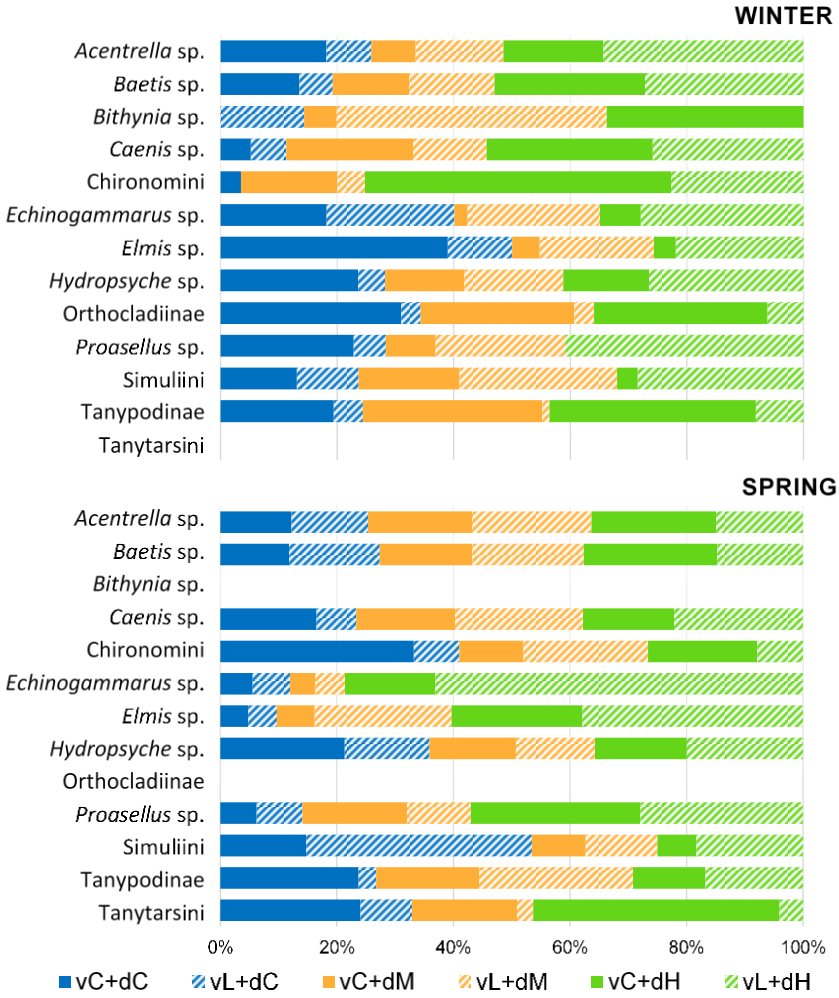

Figure 2.5. Proportion (%) of drift ratio (mean) for each treatment given for the common taxa found in both seasons, discriminated for winter and spring. Bars filled with light/dark colour indicate regular/low flow velocity, respectively. Different colours indicate oxygen levels tested (blue - normoxia; orange – medium DO depletion; green – higher DO depletion).

2.4. Discussion

Water scarcity and low dissolved oxygen concentration in the water column have been reported as two main stressors impacting lowland streams and Mediterranean systems, affecting macroinvertebrates' community structure and functioning (Acuña et al. 2005, Boix et al. 2010, Connolly et al. 2004). Indeed, in our experiment water scarcity (physical stressor) and oxygen depletion (chemical stressor) showed significant single and combined effects in the macroinvertebrate community, confirming our main predictions. Slower water flow velocity did intensify additional hypoxia conditions effects on macroinvertebrate community resulting into a greater drift behaviour with the interacting stressors.

2.4.1. Single effects of low flow velocity and oxygen depletion on macroinvertebrate community drift

The downstream transport of benthic invertebrates in the water column (drift) can occur actively or passively (Brittain and Eikeland 1988) and has been related to a variety of biotic and abiotic factors, including life-cycle stage, current/discharge (Gibbins et al. 2010, Poff and Ward 1991, Schülting et al. 2016), water chemistry and contamination (Araujo et al. 2016, Beketov and Liess 2008). Our study revealed an overall significant decrease in macroinvertebrate community drift behaviour with slower flow velocity. Given that passive drift occurs when organisms accidently lose hold from the substrate due to hydraulic stress, it seems apparent that this result should be attributed to organism's lower passive drift (i.e. involuntary) under low flow velocity conditions, favouring their permanence in the flume. So it seems likely that a low flow velocity threshold has not been reached here, point from which an active drift (voluntary behaviour) should be triggered (Brittain and Eikeland 1988), since other studies have reported an increase of active drift with periods of low flow (e.g. Minshall and Winger 1968, Poff and Ward 1991). However, they had other inherent confounding factors acting simultaneously, such as temperature, type of sediment, community density and water chemistry (review in Dewson et al. 2007), which was not the case in our experiment, since our low flow treatment was only tested for decrease in physical current, maintaining the overall physical-chemical parameters similar to the control.

Despite some individual taxa had actually increased their drift under low flow velocity (e.g. Simuliini), the majority of community contributed to a decreased drift pattern. Specific taxa such as Ephemeroptera (*Acentrella* sp. and *Baetis* sp.) and Diptera (Chironomini, Orthocladiinae and Tanipodinae) manifested particularly lower drift behaviour under reduced flow velocity. In fact, while a decrease in flow velocity is considered a stressor to rheophilic taxa, it can be expected to enhance the habitat suitability for other limnophilic taxa which prefer slow current velocities, such as the case of *Baetis* sp*., Caenis* sp*., Echinogammarus* sp. and most Diptera (Schmidt-Kloiber and Hering 2015, Tachet et al. 2000) that were found in our experiment.

Regarding oxygen depletion effects, as expected, macroinvertebrate community drift rates were greater under higher levels of DO depletion (and correlated conductivity increase) in both seasons. In accordance, previous studies showed the stressful or even lethal effects of higher hypoxia on macroinvertebrates and their avoidance behaviour to such conditions (e.g. Burnett and Stickle 2001, Connolly et al. 2004, Graeber et al. 2013, Hanazato and Dobson 1995, Pardo and García 2016) suggesting that drift will occur at low DO conditions, as was found in this study. This finding further suggests that drift was an active behaviour of avoidance to low dissolved

oxygen conditions, even though we could not fully discard the fact that a portion of individuals might have entered the drift due to an involuntary displacement (passive drift) indirectly promoted by physiological constrains under hypoxia (Burnett and Stickle 2001, Connolly et al. 2004) disabling organisms to react actively. Nevertheless, the tolerance of an organism to hypoxia and its threshold values can vary considerably (Ekau et al. 2010) since they are species-specific, and it depends also on the life stage. In our study, contrary to the overall community trend, taxa such as *Echinogammarus* sp., *Proasellus* sp., *Elmis* sp. and Simuliini drifted less under the effect of oxygen depletion and this might be related with some physiological adaptations and specific respiratory traits. For instance, for oxygen uptake these taxa use plastron as respiratory mode (Tachet et al. 2000) which may help satisfy their respiratory demands by providing a thin layer of atmospheric air along their body surface, which serves as an external lung (Flynn and Bush 2008) and might confer them a potential advantage under short-term hypoxia. Moreover, some organisms are able to sustain an aerobic metabolism during acute exposure to hypoxia by efficiently use their respiratory pigments, increasing blood flowing to respiratory organs, augmented ventilation rates, or even by combining several mechanisms (Burnett and Stickle 2001). However, we could not confirm this since the specific mechanism behind this behaviour was not tested in our experiment.

Overall, our results for drift behaviour with increasing oxygen depletion indicate that, from the management point of view, it is crucial to restrict anthropogenic organic inputs into the system, as these will potentially increase water hypoxia conditions.

2.4.2. Combined-stressors effects on macroinvertebrate drift – low flow velocity × oxygen depletion

One of the first responses of macroinvertebrate communities to poor water quality is an increase in drift rates (Araujo et al. 2016, Brittain and Eikeland 1988, Elbrecht et al. 2016). Our findings showed that, even though the individual stressors low-flow velocity (physical stressor) and low dissolved oxygen concentration (chemical stressor) had opposing effects on invertebrate drift ratio, the combined effect was higher than the calculated additive effect and thus, their interaction effect was amplified (+ synergistic *sensu* Piggott et al. 2015a) (Fig. 2.3). It was possible to observe that adding a secondary stressor (oxygen depletion) led to a reversion of the relative effects of the levels of the first stressor (low flow velocity), even though the response to interacting stressors was always higher than the response to an isolated stressor. Hypoxia conditions changed the effect of low flow velocity by shifting its negative effect on drift rates to a positive effect, *de facto* increasing drift.

In spite of the preference of some taxa for slow flowing waters (i.e. *Echinogammarus* sp., *Proasellus* sp*., Baetis* sp.; Tachet et al. 2000) and the ability to cope with oxygen deficit of others (i.e. Orthocladiinae and Tanitarsini; Tachet et al. 2000), the conjugation of low flow with higher hypoxia levels induced a positive synergistic drift response of the community. This type of amplified reaction is not uncommon and was previously found when testing total drift propensity to another physical (i.e. sediment) and chemical (i.e. nutrients) stressors interaction (Piggott et al. 2015b). The positive synergistic effect detected for any pair of levels of the two stressors seems to indicate that one stressor reduces the tolerance to the other stressor (Brown et al. 2013, Hanazato and Dodson 1995, Piggott et al. 2015b). Pardo and Garcia (2016) recognized that oxygen and flow reduction triggered shifts in macroinvertebrate community structure with the decreasing of rheophilic taxa, sensitive species (i.e. EPT), and taxa with preferences for higher aeration in the water and high concentrations of dissolved oxygen. Nevertheless, indirect effects could have also occurred. It is well established that when flow is limited, additional oxygen deficit will worsen water quality by directly changing physical-chemical parameters (García and Pardo 2016, Justus et al. 2012), such as pH and conductivity, and indirectly altering biological responses such as biota metabolism and behaviour (Burnett and Stickle 2001). Even though our findings revealed an evident effect of oxygen depletion in the displacement of organisms, we were not able to fully separate the effect of dissolved oxygen reduction from the increase in conductivity inherent to the addition of sodium sulphite into the water. On the other hand, a reduction in DO with increasing conductivity often occurs with organic loads (Daniel et al. 2002, Ortiz et al. 2005), and thus the addition of sodium sulphite to our experimental design could be seen as a proxy for several stressors, *per se*. This could only increase the effect of the addition of sodium sulphite on the response variable, even so, the reduction in water availability interacted synergistically with the addition of sodium sulphite, i.e., the physical impact enhanced synergistically the impact of a proxy for multiple interacting stressors. This strengthens the impact of our results and how anthropogenic impacts are going to be enhanced by climatic changes, especially in Mediterranean regions where the future climatic changes are expected to exacerbate the natural water scarcity. Besides, even though the drift of some aquatic invertebrates may be affected by an increase in water conductivity promoted by salt addition (Blasius and Merritt 2002), the negative impacts of sewage contamination over short time on these organisms were previously indorsed to be more likely due to reductions in DO levels than to toxicant effects (Lowell and Culp 1999).

2.4.3. Seasonal effect on macroinvertebrate drift responses to stressors

As initially hypothesized, macroinvertebrate drift rate patterns varied between seasons. Although, the drift rate tended to have the same direction of response to both stressors in winter and spring, the drifting taxa were different. These findings were expectable, since responses are species-specific and are closely linked to organisms' life-cycles, feeding habits and reproductive timings. Within a natural system, macroinvertebrate community's structure and abundance, naturally change over a temporal vector with seasonal changes in hydrology, temperature and photoperiod (Dudgeon 1990, Rincón and Lobón-Cerviá 1997, Schreiber 1995, Williams 1990).

In our experiment, we found a trend for higher differences between observed and predicted effects of stressors interaction in winter. In fact, the synergy of different levels of oxygen depletion (dM and dH) with low flow velocity varied between seasons. Genkai-Kato et al. (2000) also observed significant seasonal differences in the respiratory behaviour of stonefly larvae when testing the effect of oxygen supply associated with different current velocities.

In our study, within a medium DO depletion, the flow velocity affected the drift response of macroinvertebrate community in winter, whereas in spring the response was the same independently of the flow tested. These findings may suggest that under water scarcity, macroinvertebrates sensibility to different DO depletion levels during spring are lower than in winter. In contrary, other studies had suggested the potential of temperature to increase invertebrate sensitivity to oxygen depletion (Pardo and García 2016, Sprague 1963, Winter et al. 1996), since the temperature raise reduce oxygen supply while increasing biological oxygen demand in the system. So, the natural increase in water temperature found in our experiment (mean increase of 6 °C) from winter to spring may not have had a relevant influence in the way that organisms coped with DO depletion. The different drift responses obtained in our experiment are more likely linked with seasonal variations in communities' sensitivities and vulnerabilities, which can change temporally during their lifespan and vary between life stages, than with temperature. For example, during winter, and although not quantified here, smaller specimens were observed than in spring, including the taxa *Baetis* sp., *Acentrella* sp. and *Hydropsyche* sp. which were very representative in all drift samples. Besides, as highlighted by Segner et al. (2014) more attention should be given to biological receptor characteristics in assessing the combined effects of multiple stressors, rather than to stressor characteristics alone. Likewise, the type of organism and level of biological organization was suggested to be important factors to have in consideration when determining and predicting multiple stressors effects (Jackson et al. 2016). Though the incorporation of species tolerances in biological

assessments of aquatic ecosystems has been considered in the past (Armitage et al. 1983, Hilsenhoff 1987, Statzner et al. 2001, Usseglio-Polatera et al. 2000), most of the taxa tolerance rankings in those studies are based on qualitative judgments, rather than on experimental mesocosm data where the variables of interest can be manipulated while controlling for confounding effects.

2.4.4. Conclusions

Our study demonstrates that when water scarcity is a prevailing condition, human-impacts causing stream hypoxia have a negative impact on macroinvertebrates community. The interacting effect of low flow velocity and severe oxygen depletion amplified organisms' avoidance triggering drift behaviour, more than the single stressors effects, thereby inducing a positive synergistic effect. This change in drift patterns has potentially strong ecological consequences to higher trophic levels. In addition, we found that seasonality also plays an important part in the effect of the combined stressors. Though temperature may have an influence in the way that organisms cope with DO depletion, possibly the different invertebrate responses are closer related with seasonal variations in communities' sensitivities and vulnerabilities due to their life cycles.

From the ecosystem management point of view, in situations where these stressors are already in action generating synergistic effects, mitigation or restoration efforts focusing in reducing a single stressor, and hypoxia in particular, may yield a larger overall benefit than foreseeable.

Consequences of water pollution and water availability on structure (and possibly functioning) of the macroinvertebrate community are expected to be stronger with the predicted increases in duration and magnitude of the low flow period due to climate change and increasing water demand. Therefore, water management in Mediterranean rivers under global change should not only focus on the conservation of natural flow regimes but also, and more importantly, avoid the additional oxygen depletion caused by organic inputs by restricting effluent permits and by reinforcing water cleaning treatments under a seasonal low flow discharge.

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Chapter 3

The effect of hypoxia and flow decrease in macroinvertebrate functional responses: a trait-based approach to multiple-stressors in mesocosms

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Calapez AR, Serra SRQ, Santos JM, Branco P, Ferreira TM, Hein T, Brito AG, Feio MJ (2018). The effect of hypoxia and flow decrease in macroinvertebrate functional responses: a trait-based approach to multiple-stressors in mesocosms. Sci Total Environ 637–638, 647–656, DOI: 10.1016/j.scitotenv.2018.05.071

3. The effect of hypoxia and flow decrease in macroinvertebrate functional responses: a trait-based approach to multiple-stressors in mesocosms

Abstract

River ecosystems are most often subject to multiple co-occurring anthropogenic stressors. Mediterranean streams are particularly affected by water scarcity and organic loads that commonly lead to a simultaneous reduction in flow and increasing depletion of dissolved oxygen.

In the present study, the single and combined effects of water scarcity (flow velocity reduction) and dissolved oxygen depletion were used to evaluate alterations of drifting macroinvertebrates on a channel mesocosm system, by employing a multiple trait-based approach.

Our main findings confirmed that the impact of the two combined stressors can be implicated in alterations of ecosystem functions as result of the changes in proportions of biological traits. Overall, our results showed that, individually, flow velocity reduction and a severe oxygen depletion promoted a shift in community traits. In more detail, biological traits describing the dispersal of organisms and their respiration showed the strongest responses. The respiration mode responded to low flow with drift increase of gill breathers and decrease of individuals with tegument, whereas dispersal was clearly affected by the combination of stressors. Resistance through eggs was higher with the single effect of flow reduction, while swimmers´ relative abundance increased in individuals that drift after exposure to the combination of stressors. Thus, while flow reduction alone is expected to specifically filter out the gill breathers and the egg producers, the combination of stressors will impact more drastically organism's dispersal and swimmers. Such changes in biological traits can result in variations in ecosystem functioning through, for example, local changes in biomass, secondary production, stream metabolism as well as resulting in biodiversity losses or alterations of its distribution patterns.

Keywords: Streams, Mediterranean, Hypoxia, Flow velocity, Trait diversity, Ecosystem functions

3.1. Introduction

Most freshwater ecosystems are exposed to multiple anthropogenic stressors and natural disturbances in such way that the potential for interactions among them is a major concern to predict ecological changes and develop effective management and restoration strategies (Sala et al. 2000, Dudgeon et al. 2006, Ormerod et al. 2010).

In the Mediterranean region, river ecosystems are particularly affected by water scarcity (Barceló and Sabater 2010). These systems present a characteristic seasonal hydrological fluctuation with natural flow reduction periods (Bonada and Resh 2013). The increasing water demand for human development and higher frequency and duration of droughts with global changes (IPCC 2014), probably exacerbate these stressor effects on biota and ultimately on ecological processes and ecosystem functioning (Dudgeon et al. 2006, Ormerod et al. 2010). Additionally, many Mediterranean watersheds are densely populated and prone to water contamination from diffuse and point sources (Paul and Meyer 2001). Less availability of water will decrease the capability to dilute the organic contaminants with increases in suspended solids, nutrient concentrations, conductivity and frequent decreases on dissolved oxygen concentrations caused by higher organic matter accumulation among other stressors (Barceló and Sabater 2010, Pardo and Garcia 2016). Thus, a flow reduction in combination with high organic/nutrient loads can easily intensify the occurrence, frequency and duration of hypoxia events in streams (Franklin 2014) and increase the interaction with other anthropogenic stressors (Arenas-Sanchez et al. 2016). Nonetheless, the outcome of these interactions among co-occurring stressors are recurrently non-additive, i.e., synergies or antagonisms, constituting ecological surprises (Darling and Côté 2008, Piggott et al. 2015) that impose difficulties to analyse, quantify and predict biota responses to multiple stressors.

Macroinvertebrates comprising a wide diversity of taxa with different levels of sensitivity to stressors have been one of the most commonly used bioindicators in ecological monitoring of rivers (Rosenberg and Resh 1992, Rosenberg et al. 2008). They are relevant elements in freshwater ecosystems, contributing to their functioning through organic matter processing and energy transfer to consumers at higher trophic levels, among other processes (Heino 2005). Several factors may influence the distribution and changes in macroinvertebrates composition. Slow current velocities can trigger different drift responses, including lower (Dewson et al. 2007), higher (Acuña et al. 2005) or no change (James et al. 2008) in abundance/density in local communities, whereas large oxygen deficits in the water have been consistently found as a deleterious factor for such organisms (Burnett and Stickle 2001, Cox 2003, Pardo and Garcia 2016, Calapez et al. 2017). Nevertheless, taxonomically based metrics are often limited in

classifying mechanisms of impact (e.g. Wooster et al. 2012), making it unlikely to identify the relative importance of different stressors. In contrast, the use of community' biological traits has often provided a mechanistic understanding of anthropogenic stressors impacts (Verberk et al. 2013). In addition, establishing classifications of species based on their attributes rather than their taxonomy is assumed to relate species directly or indirectly to ecosystem functioning (Feio and Dolédec 2012, Webb et al. 2010, Logez et al. 2013), making it possible to compare systems and assemblages composed of different species pools (Lamouroux et al. 2002). Biological traits include life histories characteristics, morphological adaptations, and physiological requirements that are inherent to an organism. So, in a given environment, successful traits will be selected whereas unsuccessful traits will be filtered out (Townsend et al. 1997). Therefore, these functional responses of the community provide a causal explanation of how environmental drivers/stressors are operating in the community and can be broadly applied across temporal and spatial scales (Poff 1997, Mcgill et al. 2006).

Biological traits have been successfully used as bioindicators of diverse anthropogenic pressures on riverine systems, including multiple stressor gradients (Diaz et al. 2008), helping to disentangle their co-occurring effects on stream ecosystem functioning (Dolédec et al. 1999; Statner and Beche 2010, Wooster et al. 2012, Mondy and Usseglio-Polatera 2013, Mondy et al. 2016, Kuzmanovic et al. 2017). Nevertheless, experimental ex-situ studies that explore in a more controlled way (i.e. mesocosm assays) the trait level response of aquatic organisms to multiple pressures, such as water scarcity and the presence of hypoxia conditions are, to our knowledge, still lacking, despite some efforts had been recently made (Calapez et al. 2017). Thus, in this study we use a mesocosm system to test the single and combined effect of flow reduction and oxygen depletion on macroinvertebrate community´s functional response. Overall, we aimed at a further understanding of which biological traits (specifically the ones that might respond directly to the stressors) would be likely to be filtered out in a scenario of increasing hypoxia during a low flow event and foresee what would be the consequences for the ecosystem functioning. In particular, we wanted to determine (a) the changes in macroinvertebrate biological trait composition that drift after exposure to stressors, and (b) which traits reveal better the single and combined effect of the stressors.

3.2. Methods

3.2.1. Experimental system and design

The experiment was conducted during spring between 17th May and 2nd June 2016 in an outdoor artificial stream system installed at School of Agriculture campus (Lisbon, Portugal) which was composed by 5 independent stainless-steel-lined channels (0.4 m width × 4 m length x 0.2 m depth) (Fig. 3.1A). Water was pumped from an *in situ* natural spring (pH = 8.04 ± 0.1, conductivity = 710 ± 25 µS/cm, DO = 8.6 ± 0.2 mg/l; n = 5, mean ± SD) into a 3000-L central reservoir and evenly delivered to the channels. Each mesocosm channel incorporated a head tank (70 L), a downstream tank (70 L) and a pump (Kripsol OK-71 B; 0.75 HP) that was set to operate in recirculation flow system in order to maintain the same experimental conditions in each mesocosm (Fig. 3.1B). At the lowermost section of each channel, an experimental area (0.4 m × 0.6 m = 0.24 m^2) covered with clean substrata (mostly gravel and pebble) was set to mimic natural stream bed. A removable drift net (500 µm mesh size) was installed at the outflow of each mesocosm channel to collect the drifted individuals.

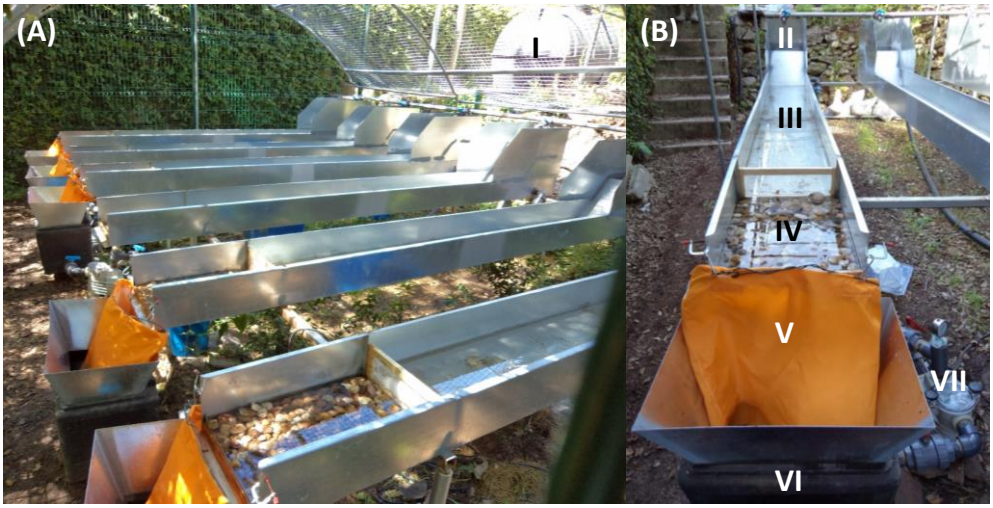

Figure 3.2. Mesocosms system used in this study. Overview of experimental system setup (A). Artificial channel with individual water recirculating system (B). I – central source reservoir with distributing pipe system, II – head tank, III – channel, IV – experimental area, V – removable drift net, VI – downstream tank, VII – pump.

Benthic macroinvertebrates used in the experiment were collected in River Jamor (a costal Mediterranean river), which is located 6.07 km west of the mesocosm facility. River Jamor is a 16.6 km long tributary of Tagus River that drains a 44.5 km^2 catchment (Tagus basin), with a lithology mainly composed by sedimentary rocks (limestone and sandstone). Each channel

received a standard load of invertebrates collected by kick-net sampling (500 µm mesh size, 25 × 25 cm frame size) from an area of about 0.25 m^2 (comparable with the 0.24 m^2 channel experimental area), taking care to sample representative pool and riffle habitats (1 m upstream for each habitat in total per sample). Macroinvertebrate standard samples were transported in a cooler and acclimatized with the water of the mesocosm system for about 24h. Afterwards, each sample were randomly transferred and acclimatized to channel conditions for at least 1 hour *prior* to the test, maintaining a water column of approximately 8 cm depth. This process was repeated for each treatment replication, so fresh macroinvertebrate samples were tested for each experiment in consecutive days.

The invertebrate samples were submitted to stressors following a factorial design of two flow velocity levels x three levels of dissolved oxygen depletion with five synchronous replicates of each treatment combination. The invertebrate direct response, as drift abundance, was measured after three hours of exposure to stressors. Taking into account a trait-based approach, drift is the real direct response to the tested stressors in a short term, since all organisms that enter the drift own biological traits that disable them towards the stressors, not providing any advantage and excluding them directly. For the remaining organisms, there was enough time to guarantee that their better adapted biological traits would be fully favoured, and so not representing a real natural situation.

By combining a regular flow velocity (vC = 0.19 ± 0.02 m/s) and a low flow velocity (vL = 0.06 ± 0.02 m/s) with normoxia condition (dC = 100% sat. DO) and two decreasing levels of dissolved oxygen (DO) in the water (dM = 65% sat. DO, mild depletion and dH = 40% sat. DO, severe depletion, respectively) we obtained six experimental treatment combinations testing the single and combined effects of the stressors: (1) regular flow velocity with normoxia - vCdC - as control treatment; (2) low flow velocity with normoxia - vLdC - as single effect of water scarcity; (3) regular flow velocity with mild DO depletion - vCdM - as single effect of mild DO depletion; (4) regular flow velocity with severe DO depletion - vCdH - as single effect of severe DO depletion; (5) low flow velocity with mild DO depletion - vLdM - as combined effect of water scarcity and mild DO depletion; (6) low flow velocity with severe DO depletion - vLdH - as combined effect of water scarcity and severe DO depletion.

A regular flow velocity (vC) was defined as control (corresponding to the average velocity measured in the WFD-oriented Spring sampling of the river type which River Jamor belongs) while the low flow velocity (vL) is observed when the system starts to contract in the dry season, in early Summer.

Mild level of oxygen depletion (dM) was chosen as the saturation in which most sensitive species actively enter the drift (Connolly et al. 2004), whereas the severe oxygen depletion (dH) was

selected to be near the threshold that starts to cause stressful conditions to some aquatic organisms (4 mg/L; ca. 45% sat. DO; Franklin, 2014) and induce a strong drift response in moderate to tolerant macroinvertebrate species. This oxygen reduction in the water can occur in streams not only as a consequence of water scarcity and flow reduction (Pardo and Garcia 2016) but also as the result of discharges of organic wastes (Misra et al. 2006).

Oxygen depletion was induced by adding sodium sulphite (Na_2SO_3) (Park et al. 2014) into the water of each mesocosm channel, following the same procedure used in a previous parallel study (see Calapez et al. 2017 for further details). As oxygen scavenger (Lewis 1970), Na_2SO_3 has been formerly used in the setting of oxygen-deficit conditions in animal and water research (Crampton 1998, Peay et al. 2006, Branco et al. 2016, Calapez et al. 2017), and was employed in the present study as a proxy for the reduction of DO in rivers due to the degradation of organic loads into the system.

Flow velocity levels were measured for each experiment with a digital water velocity meter (model FP101, Global Water Instrumentation, USA) and temperature (°C), pH, conductivity (µS/cm), and total dissolved oxygen (%DO) were monitored in all mesocosm channels throughout the experiment (n=4) with multiparametric water probes (HANNA, model HI 9812-5 and HI98193).

After treatment exposure, drift net contents, as well as the remaining individuals in each channel, were collected and preserved with ethanol (96%) for later sorting and counting. Drifting and non-drifting macroinvertebrate samples were processed individually to enable assessing the overall initial tested community.

3.2.2. Trait composition

Macroinvertebrate identification was done to the lowest possible taxonomic level (i.e., genus and species level), with the exception of Diptera (identified to family, subfamily or tribe level) and Oligochaeta (family). Traits were attributed to taxa (mainly genera) following trait collection information described in Tachet et al. (2010), which was available for 99.9% of our total macroinvertebrate collection.

A set of 4 biological traits with a total of 15 trait categories were chosen *a priori* (see Table 3.1) that are expected to directly respond to the designated stressors in this study, including biological characteristics that provide the organisms with the ability to cope with oxygen depletion and variations of flow regime in a short-term: dispersal, respiration, resistance form and locomotion (Statzner and Beche 2010). This selection was kept to biological traits, because these reflect the ability of organisms to cope with environmental constraints and consequently

their survival abilities, emphasizing their functional role in the ecosystem (Verberk et al. 2013, Mondy and Usseglio-Polatera 2014).

Table 3.2. Biological traits with corresponding categories selected for this study.

Biological traits	Category
Dispersal	Aquatic passive
	Aquatic active
	Aerial active
Respiration	Tegument
	Gills
	Plastron
	Spiracle (aerial)
Resistance form	Diapause or quiescence
	Cocoons
	Eggs/statoblasts/gemmules
	None
Locomotion	Swimmer
	Crowler
	Burrower (epibenthic)
	Temporarily attached

3.2.3. Data analysis

The affinity of each taxon for each trait category was quantified using the fuzzy-coding procedure (Chevenet et al. 1994), by standardizing trait categories (e.g., aquatic passive, aquatic active, aerial active) to sum 1 within each trait (e.g., dispersal). This method ensured that all taxa had the same weight in further analyses. Trait-by-samples arrays were further computed as the cross-product between the invertebrate taxa abundance ln(x+1) transformed and the standardized trait profile (e.g. Gayraud et al. 2003). This resulted in trait-by-samples arrays expressing the relative abundance of each trait category that drifted in each mesocosm channel after treatment exposure.

To guarantee that the same trait composition was being represented evenly in all treatments before the exposure to stressors, significant differences ($p<0.05$) for initial macroinvertebrate relative trait abundance between treatments were tested using permutational multivariate analysis of variance (PERMANOVA; 9999 permutations) for the different treatments (n=6) based on a Bray-Curtis similarity matrix.

A between-group Fuzzy Correspondence Analysis (FCA; Chevenet et al. 1994) was performed to determine the proportion of variance explained by the difference among groups constituted by taxa that drifted after exposure to vC/vL water velocity and to dC/dM/dH oxygen depletion condition.

Significant differences ($p<0.05$) for drifted macroinvertebrate trait abundance or each stressor and their interaction were tested using PERMANOVA (9999 permutations based on a Bray-Curtis similarity matrix) for the two fixed factors “flow velocity” and “depletion of DO” with two (vC and vL) and three levels (dC, dM and dH) respectively, followed by pair-wise post hoc tests.

Trait categories that contributed for the differences ($p<0.05$) between treatments were assessed by performing a Kruskal-Wallis test. Trait categories showing significant differences among treatments were afterwards tested using pair-wise post hoc Dunn´s test.

Statistical and graphical analyses were performed using R freeware (R Core Team 2016) employing the packages “ade4” and “vegan” (Thioulouse et al. 1997, Chessel et al. 2004, Dray and Dufour 2007, Dray et al. 2007) and PRIMER 6 & PERMANOVA+ software (PRIMER-E Lda, Plymouth UK) (Anderson and Robinson 2001).

3.3. Results

3.3.1. Experimental conditions and macroinvertebrate trait samples

During the experiment, water flow velocity was reduced in about 70% from vC to vL (from 0.19 m/s ± 0.005 SD to 0.06 m/s ± 0.004 SD, respectively), while dissolved oxygen was decreased in about 35% for dM and 61% for dH (from 99% sat. ± 5.9 SD in dC to 65% sat. ± 4.5 SD in dM to 39% sat. ± 22.1 SD in dH) (Fig. 3.2).

Parameters monitored during the experiment displayed in Table 3.2, showed that temperature and pH presented similar values between the different treatments (ranging from 20.3 °C to 23.7 °C for temperature and 7.9 to 8.7 for pH), while an increase in conductivity accompanied the decrease of dissolved oxygen in the water, showing an increasing gradient from dC to dM to dH treatments.

Macroinvertebrate taxonomic identification resulted in the classification of 35 different taxa comprising a total of 36 925 individuals. Among these, Ephemeroptera, namely *Baetis* sp., *Acentrella* sp. and *Caenis* sp. were the most common and abundant taxa (86.9%) in all initial samples used to test the stressors, but other representative taxa also included Trichoptera

(*Hydropsyche* sp. and *Hydroptila* sp.; 3.4%), Diptera (Simuliini, Tanypodinae and Tanitarsini; 2.9%) and the crustacean *Echinogammarus* sp. (5.6%).

Traits categories were attributed to 87.5% from the total number of taxa identified in this study.

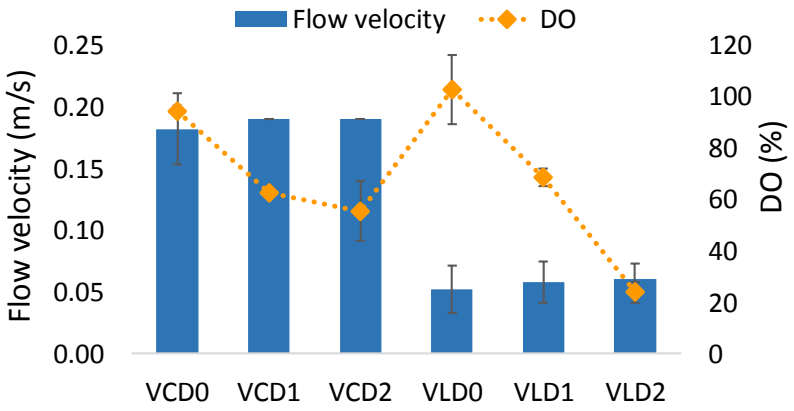

Figure 3.3. Mean flow velocity ± SD (bars; m/s) and mean dissolved oxygen concentration ± SD (lines, %) during the experiment (n = 4) for the different treatment combinations. vCdC - control; vCdM – mild DO depletion effect; vCdH – severe DO depletion effect; vLdC - low flow velocity effect; vLdM - combined effect of low flow and mild DO depletion; vLdH - combined effect of low flow and severe DO depletion.

Table 3.2. Physical-chemical parameters (mean ± SD) measured during the experiment (n=4) for each treatment combination. vCdC - control; vCdM - mild DO depletion effect; vCdH - severe DO depletion effect; vLdC - low flow velocity effect; vLdM - combined effect of low flow and mild DO depletion; vLdH - combined effect of low flow and severe DO depletion.

Treatment	Temp (°C)		pH		Cond. (µS/cm)	
	Mean	SD	Mean	SD	Mean	SD
vCdC	23.7	0.8	8.2	0.07	532.0	23.3
vCdM	21.8	0.3	8.7	0.06	1076.0	47.7
vCdH	22.7	0.1	8.7	0.03	1370.2	36.3
vLdC	21.8	0.7	7.9	0.06	503.0	40.2
vLdM	20.3	0.4	8.7	0.01	1243.5	50.3
vLdH	23.8	0.2	8.6	0.03	1279.7	23.7

Results given by PERMANOVA testing the differences between samples of initial relative trait abundance *prior* to the tests, showed no significant differences (Pseudo-F=1.319, p-value=0.171), confirming that all the different treatments were applied to an identical initial trait "pool". The trait categories most represented in such initial trait "pool" were aquatic passive dispersal (19.1%), none resistance form (17.7%), respiration by gills (15.2%) and locomotion by

crawling (18.9%), whereas the less represented comprised aquatic active dispersal (1.5%) respiration by plastron (0.3%), diapause as resistance form (1.3%) and burrower as locomotion form (0.3%).

3.3.2. Traits response to flow velocity and oxygen depletion

Macroinvertebrates trait relative abundance was segregated by the Fuzzy Correspondence Analysis (FCA) based on stressors exposure. The FCA performed on drifted macroinvertebrate trait relative abundance yielded a first and second axis that explained 39.9% and 18.0% of the total variability, respectively, with significant differences between treatments (explained variance: 35.7%, simulated p-value: 0.001, Fig. 3.3). The first FCA axis segregated trait samples exposed to low flow velocity (vL) from trait samples exposed to the control flow velocity (vC) (Fig. 3.3a). The second axis separated traits subjected to a severe DO depletion (dH) from the ones in a normoxia condition (dC), and although traits exposed to a mild DO depletion (dM) did not display a clear separation from the remaining treatments, it is evident a trait gradient regarding the oxygen condition (dC to dM to dH) (Fig. 3.3b).

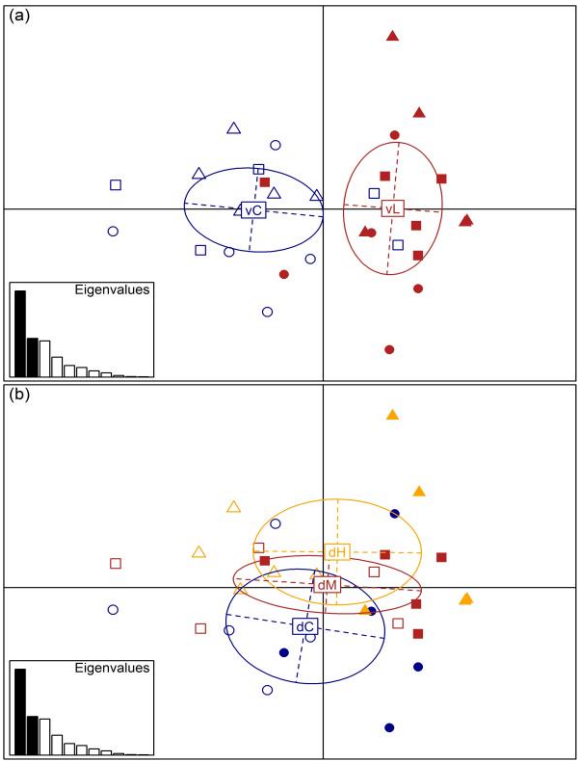

Figure 3.3. First-two axes of a between-group Fuzzy Correspondence Analysis performed on drifted macroinvertebrate trait relative abundance, in which ellipses show sample ordination grouped by (a) flow velocity treatment: vC – velocity control (open symbols), vL – lower velocity (filled symbols); and (b) by oxygen condition: dC – control (circles), dM – mild DO depletion (squares), dH – severe DO depletion (triangles). Histogram of eigenvalues are shown. Ellipses encompass 40% of the replicates along the axis for readability.

The PERMANOVA (Table 3.3) showed that both single stressors had a significant effect on traits relative abundance of drifting individuals. When comparing to the control (dC), traits of drifting individuals were significantly different under the highest DO depletion level (dH) (PERMANOVA pair-wise tests; t = 1.763, p = 0.021). The exposure to a lower flow velocity (vL) resulted also in different trait relative abundance compared to the control (vC) (PERMANOVA pair-wise tests; t = 2.156, p = 0.005)

Table 3.3. Effects of single and interacting factors (oxygen and flow velocity) on macroinvertebrate trait relative abundance (PERMANOVA, 9999 unrestricted permutations based on Bray-Curtis similarity matrix). Significant p-values (p<0.05) are displayed in bold. Pairwise tests overall results are also shown. dC – normoxia, dH – severe DO depletion, vC – regular velocity, vL – low velocity.

Source of variance	Pseudo-F	p-value	Pairwise tests (significance p<0.05)
Oxygen	2.273	**0.043**	dC ≠ dH
Velocity	4.648	**0.005**	vC ≠ vL
Oxy × Velocity	0.805	0.574	

3.3.3. Stressors effects on trait categories

Kruskal-Wallis tests revealed significant differences (p<0.05) between treatments in 7 of the 15 trait categories selected (Table 3.4). For these, trait relative abundance for each treatment and corresponding differences are displayed in Figure 3.4. Overall, no differences were observed between different levels of DO depletion in combination with the flow velocity decreasing treatment, i.e., vLdM was not significantly different from vLdH treatment for any of the trait categories (Fig. 3.4).

Dispersal was the most affected biological trait, displaying significant differences for all categories between treatments (Table 3.4). Aerial dispersal was significant lower (p<0.05) after exposure to the combined effect of low velocity and both levels of oxygen depletion (vLdM and vLdH) when comparing to the control (vCdC) (Fig. 3.4). An identical result was obtained for the aquatic active dispersal in which trait abundance was also lower in the combined low velocity and DO depletion treatments relative to the control (Fig. 3.4). Also, stressors combined effect induced an aquatic active dispersal decrease relatively to the single effect of mild DO depletion (vCdM) and to single effect of low velocity (vLdC).

Table 3.4. Kruskal-Wallis test results concerning the differences between treatments combination for each trait category. Significant p-values (p<0.05) are displayed in bold.

Trait	Category	Chi-Square	p-value
Dispersal	Aquatic passive	15.508	**0.008**
	Aquatic active	15.648	**0.008**
	Aerial active	13.124	**0.022**
Respiration	Tegument	19.106	**0.002**
	Gills	15.818	**0.007**
	Plastron	8.928	0.112
	Spiracle	7.194	0.207
Resistance form	Diapause or quiescence	9.821	0.080
	Cocoons	7.021	0.219
	Eggs/statoblasts/gemmules	14.280	**0.014**
	None	8.025	0.155
Locomotion	Swimmer	12.241	**0.032**
	Crowler	7.039	0.218
	Burrower	4.539	0.475
	Temporarily attached	5.279	0.383

Within the aquatic passive category, trait abundance was higher with the combined effect of low flow and both levels of DO depletion (vLdM and vLdH) relative to the control (vCdC) and also relative to single effect of dH oxygen depletion (vCdH) and single effect of low velocity (vLdC) (Fig. 3.4).

Regarding respiration, the category gill had a higher relative abundance under low velocity (vL) than in the control (vC), independently of the level of DO depletion. In this category there was a single effect of low velocity (vLdC), as well as a combined effect of low velocity and both levels of DO depletion, relatively to the control (vCdC) (Fig. 3.4). Tegument relative abundance showed a reverse pattern, i.e., tegument trait abundance was lower with both low velocity treatments (vL) than in the velocity control treatments (vC). Thus, significant differences were observed for the single effect of low velocity (vLdC) and for the combined effect of the stressors (vLdM and vLdH) compared to the control (vCdC) (Fig. 3.4).

Concerning the resistance form, relative abundance for the egg category was significantly higher (p<0.05) for the single effect of low velocity (vLdC) and the combined effect of low velocity with mild DO depletion (vCdM), relative to control (vCdC) (Fig. 3.4).

Finally, for the swimmer trait category, relative abundance was significantly higher for the combined effect of low velocity and mild DO depletion (vLdM) relative to the control (vCdC) (Fig. 3.4).

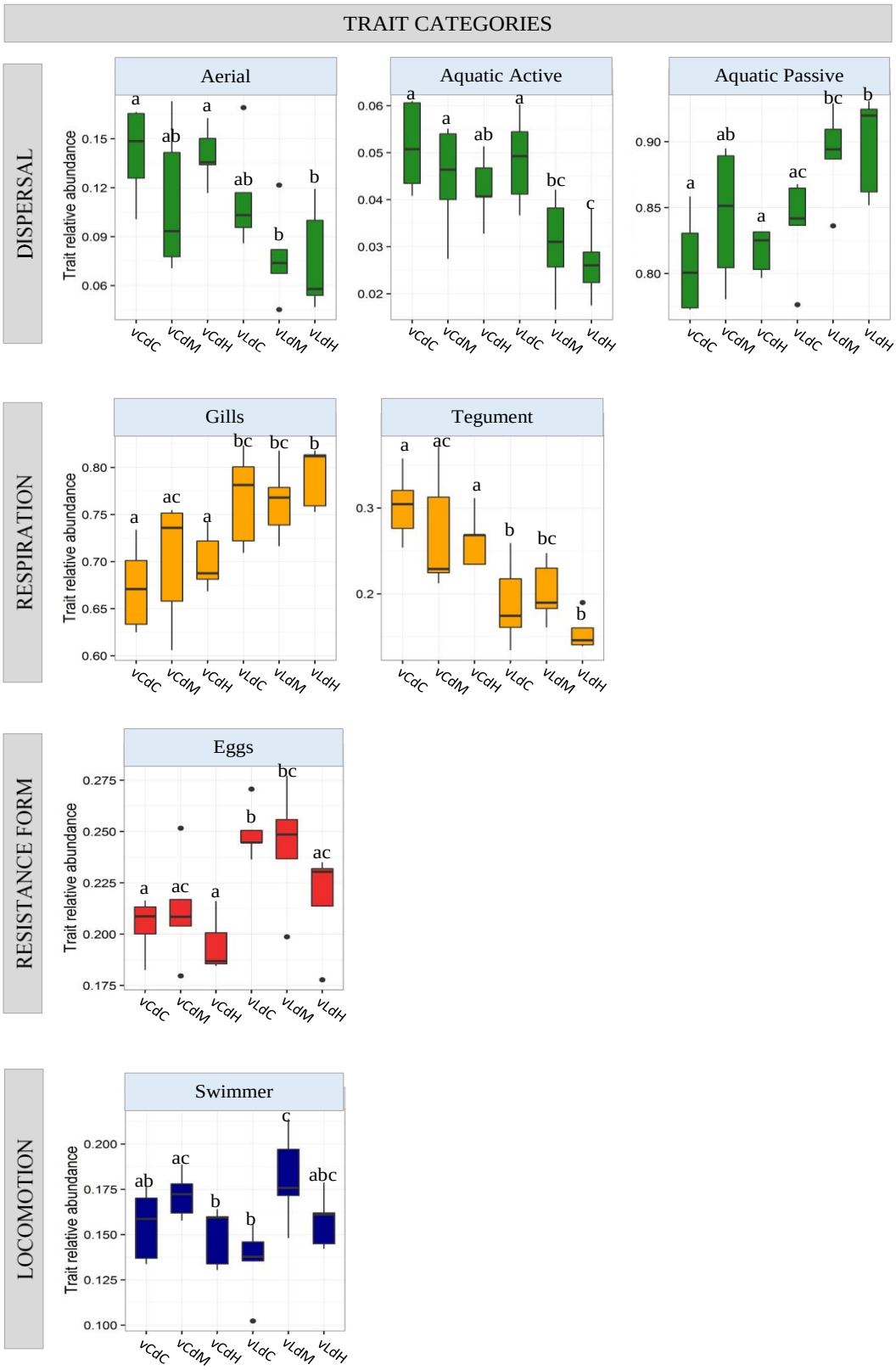

Figure 3.4. Box-plots expressing trait relative abundance for the categories that showed significant differences between treatments (Kruskal-Wallis test). Significant differences ($p<0.05$) given by pair-wise post hoc test (Dunn´s test) among treatments are represented by different letters. vCdC – control treatment, vCdM – single effect of mild DO depletion, vCdH – single effect of severe DO depletion, vLdC – single effect of low flow, vLdM – combined effect of low flow and mild DO depletion, vLdH – combined effect of low flow and severe DO depletion.

3.4. Discussion

Our main findings confirmed that the decrease of flow velocity and oxygen depletion affected macroinvertebrate communities´ drift response, promoting changes in biological traits relative abundances. Various studies have been describing water scarcity and oxygen deficits as two significant pressures affecting the ecosystems of Mediterranean rivers and macroinvertebrate communities (e.g. Acuña et al. 2005, Boix et al. 2010, Calapez et al. 2017, Garcia and Pardo 2016, Pardo and Garcia 2016). Yet, we went further, by showing that the impact of the two combined stressors (flow reduction and oxygen depletion) are beyond community level and can potentially affect ecosystem functions promoted by changes in biological traits of invertebrates. Trait approaches are based on well-defined and measurable properties of the organisms that influence their fitness (McGill et al. 2006) and ecosystem functioning (Tilman 2001).

In this study, the pool of selected traits responded strongly to the effect of single stressors - though differences observed could reflect some within-group variance for some of the trait proportions, rather than "real" differences between groups (Warton et al. 2012) - and no significant interaction was detected at this response level. But when considering the response at community composition level (i.e. taxon relative abundance), Calapez et al. (2017) found a synergistic interaction effect of both stressors, which seems to have faded when testing the traits assemblage with an identical experimental design in the present study. Accordingly, it was previously found that biological trait composition could be less sensitive than taxonomic composition and abundance to specific factors (e.g. seasonal variations; Bêche et al. 2006), and that weak changes in traits but rather large changes in taxonomic composition could be produced by climate change impacts (Bonada et al. 2007). Despite the above, when looking for each individual trait category, there was an obvious overall effect of the stressors acting in combination. This underlines that despite a single trait category could be used as an indicator of a certain set of pressures acting in the ecosystem, the evaluation of manifold biological key traits assembled (e.g. Mondy et al. 2016) may still be important to assess a more accurate impact of multiple-stressors in the system. Moreover, sensitivities and tolerances of individual taxa to the water scarcity effects seems to be determined by complex combinations of biological traits (Brooks et al. 2011).

The array of functions provided by a community should be mainly dependent on the diversity of key functional traits and the diversity of species that express them (Díaz and Cabido 2001). Functional trait diversity has significant implications in the capacity of benthic assemblages to withstand and recover from disturbances and to react to environmental changes (Poff et al.

2006). Here, single effects of flow reduction triggered alterations in proportions of specific traits in the drift (i.e. respiration and resistance form), with implications in trait diversity losses. Accordingly, reduced macroinvertebrate functional trait diversity has previously been attributed to hydrological drying events with drastic stream flow reductions (Schriever et al. 2015). A lower functional diversity suggests also lower ecological redundancy (Walter 1992), so if stream hydrology will include higher frequency and duration of drought as foreseen (IPCC 2014), unique traits could be at risk to be lost in this scenario, leading to a possible decreased productivity and disturbances of ecosystem processes.

Biological traits describing the dispersal of organisms and their respiration showed an overall stronger response, in which respiration mode responded to flow velocity decrease, whereas dispersal was clearly affected by the stressor's combination. These are two important features of organisms and a change in their proportion within the community can have major implications in ecosystem functions. Dispersal traits are related to the ability of the invertebrates to colonize different habitats and recolonize streams after disturbance, and also have important consequences for gene flow and evolutionary divergence processes (Bilton et al. 2001). Drifting organisms displayed a lower proportion of individuals with active dispersal (aerial and aquatic) and a higher proportion of individuals with aquatic passive dispersal when testing stressors in combination. Transposing to a real natural riverine environment, this could mean that the first organisms to respond by drifting from a given river reach under decreased water flow and oxygen deficits are those with less ability to endure water current and to search for a different habitat. Thus, this could also mean they will be the first colonizers of new areas: simpler individuals without the ability to actively disperse in the water or to fly. Ultimately these changes in drifting organisms must likely result in a disequilibrium in biomass along the river continuum and changes in secondary production (Allan and Castillo 2007), as well as trait diversity losses which give rise to a wide range of reasonable impacts of disappearance on ecosystem function (Cardinale et al. 2012). In addition, it has been suggested that dispersal is a crucial factor for successful (re)colonization in cases of stream rehabilitation/restauration (Brederveld et al. 2011), so the inability of dispersing actively will place an even greater difficulty for the dissemination of organisms, either to upstream or to another suitable stream reaches nearby (Nilsson et al. 2002).

As for respiration mode, this trait may have implications in the ecosystem metabolism and define the body size of individuals (Tachet et al. 2010). Organisms that use gills and tegument as respiration mode responded in opposing ways to the single effect of low flow and to the combined effect of low flow with both levels of DO depletion. These results indicate that the respiration mode of organisms can discriminate flow velocity decreasing among additional

hypoxia conditions and can be used as an early warning signal of ecosystem problems. Accordingly, respiration through tegument and through gills amid other traits, had been previously disclosed as a potential indicator for detecting effects of water abstraction on stream invertebrates (e.g. Lange et al. 2004). Whereas the short-term response of gill breathing individuals was to increase drift when exposed to both single and combined stressors (i.e. flow velocity decrease alone and in combination with both hypoxia conditions), the response of individuals with tegument was to drift less in such settings. These results indicate that the flow velocity is the major stressor triggering the respiratory response of organisms. Invertebrates with tracheal gills may be able to tolerate low DO levels because of their larger respiratory surface area in relation to body size (Dodds and Hisaw 1924), but are very susceptible to flow reduction via water quality deterioration (e. g. increased conductivity, temperature, nutrient concentration, suspended sediments) (Dewson et al. 2007). As for tegument breeders, previous studies had also found that families with tegument respiration appeared unaffected by water withdrawals (e.g. Brooks et al. 2011). In fact, a greater occurrence of taxa with tegument respiration have been closely associated to areas with modified flow regimes and other human impacts (Charvet et al. 2000, Tomanova et al. 2008). Thus, these results point to the assumption that in a real situation, macroinvertebrate communities under water scarcity and oxygen deficits will be represented by more primitive and smaller sized tegument breather individuals (Tachet et al. 2010) without the ability to regulate oxygen uptake and tolerate a wider range of DO conditions regardless of flow fluctuations (Golubkov et al. 1992). In terms of future ecosystem implications, this could mean, once more, a significant loss of biodiversity and consequently a selective reduction of ecosystem functions promoted by the gill breathers' category.

Resistance traits allow the organisms to endure the effect of a complex set of deleterious or even lethal factors occurring during an unfavourable period. Individuals that use eggs as resistance strategy responded to single effect of low water velocity and to combined effect of low velocity with the mild oxygen depletion by increasing the drift. Previous studies have addressed the importance of the production of resistance forms such as eggs to partly explain the community recovery after a dewatering event in intermittent streams (Datry et al. 2016, Larned et al. 2007, Tronstad et al. 2005). These protective structures of resting forms have morphological and physiological characteristics to maintain a good viability level until the end of the harsh environmental conditions. So, our results seem to indicate that organisms using such resting forms (i.e. eggs) will be filtered out from a habitat where water scarcity and hypoxia are prevailing conditions, which may have consequences in community potential recovery in short and long term, jeopardizing biodiversity. As a consequence, biodiversity loss may reduce

the efficiency by which ecological communities capture biologically essential resources, produce biomass, decompose and recycle essential nutrients (Cardinale et al. 2012)

3.4.1. Conclusions

Functional trait diversity can provide insights into ecosystem function further than those provided by species diversity measures (Suarez et al. 2017), and given that our results showed responses for all the selected traits, it was evidenced that these represent important biological features to have into consideration when addressing the impact of water scarcity and oxygen deficits in Mediterranean rivers.

While flow reduction alone will specifically filter out the gill breathers' individuals as well as the ones that use eggs as resistance form in response to adverse conditions, the combined stressors will impact more drastically organism's passive dispersal and locomotion by swimming.

Overall, this study showed that low water velocity and oxygen depletion conditions acting in isolation and combined can induce a loss of trait diversity in the macroinvertebrate communities, which may reduce the provision of ecosystem services and the ability of a stream to recover when normal situations are re-established (Elmqvist et al. 2003, Mumme et al. 2015).

From the ecosystem management point of view, the use of taxonomic data was found as a useful tool in assessing multiple-stressors effects (Calapez et al. 2017). In spite of taxa abundances have proven to be more sensitive in detecting the synergistic effects of low flow and oxygen depletion (Calapez et al. 2017) when compared with organisms´ multiple traits proportions as was found in this study, we consider that the use of some key trait categories would also constitute a valuable contribute to detect specific alterations in the ecosystem while providing an indirect link to ecosystem functioning.

In the context of climatic change scenarios, which predict higher hydrological disturbances, namely increasing water scarcity and water pollution with inherent oxygen deficits, our experimental study provides new insights how the functional characteristics in river systems maybe be substantially affected and conversely the ecosystem functioning limited. Mitigation measures should focus on controlling DO deficits of anthropogenic nature such as decreasing organic loads permits, especially during hydrological contracting phases, and some local efforts in restoration may go through generating physical turbulence areas *in stream* to improve water reaeration rates, especially at more local scale in stream reaches impacted the most.

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SECTION III

RESPONSES OF MICROBIOTA AND PRIMARY CONSUMERS

Chapter 4

Sewage contamination under water scarcity effects on stream biota: biofilm, grazers and their interaction

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4. Sewage contamination under water scarcity effects on stream biota: biofilm, grazers and their interaction

Abstract

One of the most common anthropogenic impacts on river ecosystems is the effluent discharge from wastewater treatment plants. The effects of this contamination on stream biota may be intensified in Mediterranean climate regions, which comprise a drought period that leads to flow reduction, and ultimately to stagnant pools. To assess individual and combined effects of flow stagnation and sewage contamination, biofilm and gastropod grazers were used in a 5-week experiment with artificial channels to test two flow velocity treatments (stagnant flow/basal flow) and two levels of organic contamination using artificial sewage (no sewage input/sewage input). Stressors' effects were determined on biofilm total biomass and chlorophyll (Chl) content, on oxygen consumption and growth rate of the grazers (*Theodoxus fluviatilis*), and on the interaction grazer-biofilm given by grazer's feeding activity (i.e., biofilm consumption rate). The single effect of sewage induced an increase in biofilm biomass and Chl-a content, simultaneously increasing both grazers' oxygen consumption and their feeding activity. Diatoms showed a higher sensitivity to flow stagnation, resulting in a lower content of Chl-c. Combined stressors interacted antagonistically for biofilm total biomass, Chl-b contents, and grazers's feeding rate. The effect of sewage increasing biofilm biomass and grazing activity was reduced by the presence of flow stagnation (antagonist factor). Our findings suggest that sewage contamination has a direct effect on the functional response of primary producers and an indirect effect on primary consumers, and this effect is influenced by water flow stagnation

Keywords: Organic load, Nutrients, Flow velocity, Grazing, Multiple-stressors, Primary productivity, Chlorophyll, Mesocosms

4.1. Introduction

River ecosystems are significantly vulnerable to aquatic pollution resulting from diverse anthropogenic sources. One of the most common point source pollution is the discharge of domestic and industrial sewage effluents (Paul and Meyer 2001) that contributes to riverine degradation with high organic loads and nutrients among other contaminants (e.g., metals, pathogens, pharmaceuticals) (Gibson and Meyer 2007, Grantham et al. 2012, Huerta et al. 2016). These pollutant inputs have been shown to propel eutrophication (Smith et al. 1999), change stream biota communities' abundance, composition, and structure (Grantham et al. 2012, Kosmala et al. 1999, Lowell and Culp 1999, Lu et al. 2016), and ultimately change ecosystem processes (Woodward et al. 2012) such as primary production (Masseret et al. 1998, Neif et al. 2017) and nutrient dynamics (Carey and Migliaccio 2009, Merseburger et al. 2005).

The deleterious effects of sewage inputs in streams and their aquatic communities are intensified under water scarcity conditions, which are common in Mediterranean climate streams (Blasco et al. 2015). Due to the reduced flow conditions and slower current velocity characteristic of these streams (Gasith and Resh 1999), dilution capacity is largely diminished and exposure periods in stagnant zones are amplified (Petrovic et al. 2011, Prenda and Gallardo-Mayenco 1996), while sewage inputs remain continuous. In fact, wastewater effluents can dominate stream discharges in Mediterranean streams during water scarcity phase, accounting to 70 to 90% of effluent in total streamflow (Canobbio et al. 2009, Carey and Migliaccio 2009). Accordingly, combined hydrological stress and nutrient enrichment commonly interact together (Nõges et al. 2016), yet these multiple-stressor effects are complex and difficult to predict because of synergies and amplifying feedbacks among stressors that act in concert generating changes on biodiversity and ecological processes (Ormerod et al. 2010) with significant effects on biological stream communities, including biofilms.

Stream biofilms are a complex microbial assemblage mainly composed by shorter life-cycle organisms such algae, bacteria, fungi, and microfauna entrenched in a polysaccharide matrix, which are involved in key fluvial ecosystem functions such as primary production, nutrient cycling, and organic matter processing (Battin et al. 2003, Guasch and Sabater 1995). Additionally, stream biofilms are an important nutritional food resource for benthic communities of higher trophic levels (Battin et al. 2003, Lear et al. 2012), including benthic invertebrates. Among these, the grazers-scrapers feed directly on biofilms and have an important role in the ecosystem due to their high abundances among feeding groups in different sections of the river continuum (Cummins and Klug 1979). Biofilm's quality as food resource can be influenced by the relative composition of their microbial elements, thus affecting primary

consumers' diversity, abundance, and distribution along the ecosystem (Feminella and Hawkins 1995).

Within lotic systems, biofilms are one of the first elements to interact with dissolved substances responding to physical (e.g., flow decreasing), chemical (e.g., nutrient enrichment), and biological (e.g., grazing) sudden alterations and therefore may be used to efficiently detect the early effects of multiple stressors on the ecosystem (Sabater et al. 2007).

Despite the increasing attention given to pollutant inputs in streams (e.g., Corcoll et al. 2015, Neif et al. 2017, Tornés et al. 2018) and to responses of decreasing flows and desiccation conditions (e.g., Corcoll et al. 2015, Matthaei et al. 2010, Pardo and García 2016), to our knowledge, pool effects on stream biota are still understudied, especially when combined with pollutants. Besides, from the river basin management point of view, knowledge on combined stressors effects by assessing the relative importance of each stressor and their impacts on biota is crucial to find suitable measures of mitigation and restoration (Ormerod et al. 2010).

Under this context, the present study aims to investigate how both stressors (sewage contamination and water scarcity) affect river biofilms and if this effect is upscaled to the following trophic level (herbivores) through direct effects on grazers and indirect effects over biofilm quality as food resource. To determine the single and interacting effects of sewage inputs and water flow stagnation on primary producers' biomass, herbivore grazers' growth, respiration, and grazing activity rate, we conducted a 5-week mesocosm experiment with stream biofilm and gastropod grazers (Theodoxus fluviatilis), manipulating flow velocity and sewage contamination. Overall, we expect that the single effect of sewage contamination will enhance biofilm biomass specifically driven by an increase in algal growth, increasing grazer's metabolism and feeding rate as well. Water flow stagnation is expected to disfavor biofilm biomass growing and indirectly decrease grazer growth and feeding activity, mostly due to a lower quantity of food and overall lower dissolved oxygen in the water column. Moreover, because we predict opposite direction effects for the two stressors, we hypothesize that the combination of flow stagnation with sewage contamination can generate an antagonistic response depending on the magnitude of the opposing factors, decreasing biofilm biomass growth and grazers' metabolism and, consequently, affecting their feeding rate by decreasing biofilm consumption.

4.2. Methods

4.2.1. Experimental design and procedure

The experiment was conducted in an indoor experimental system consisting of a set of 14 modular mesocosm structures, each one composed by and independent polymethyl methacrylate channel (150 cm long × 10 cm wide × 12 cm high) and a 27L capacity water reservoir equipped with a submergible water pump (EHEIM compact 3000, Germany) operating in a flow-recirculating mode (Fig. 4.1a; more detail *in* Elias et al. 2017). The experimental system was set in an acclimatized room with mean temperature of 16.8±3.7°C and with a daily 12h:12h light/dark photoperiod cycle simulated by fluorescent lamps (Lumilux—8000 K; Osram, Germany). Each channel was provided with 25L of dechlorinated tap water, previously filtered through 5-µm wound polypropylene and active carbon filters, and water depth was kept at 6 cm. In each channel was set a grazing test area (28 cm × 10 cm) delimited by nets (0.2 cm mesh size) (Fig. 4.1).

To assess biofilm and grazers' response to flow cessation and sewage contamination single and combined effects, we manipulated two flow velocity treatments (Fig. 4.1): a basal flow as control (F; 0.05 m/s) and no flow to mimic summer flow stagnation conditions (nF; 0.00 m/s); and two levels of organic/nutrient contamination using artificial sewage: no sewage input as control (nS) and sewage input to mimic an effluent discharge (S). Treatments were randomly assigned to the 14 mesocosm channels in a full-factorial 2 × 2 design (with 3 replicates for treatments without sewage contamination and 4 replicates for treatments with sewage contamination) resulting in 4 different combination treatments: F+nS (control); F+S (single effect of sewage contamination); nF+nS (single effect of water stagnation); nF+S (combined effect of water stagnation and sewage contamination).

Synthetic sewage (OECD 2001) was used as proxy of a wastewater effluent (per litter of tap water: peptone 160 mg; meat extract 110 mg; urea (CH_4N_2O) 30 mg; anhydrous dipotassium hydrogen phosphate (K_2HPO_4) 28 mg; sodium chloride (NaCl) 7 mg; calcium chloride dehydrate ($CaCl_2.2H_2O$) 4 mg; magnesium sulfate heptahydrate ($Mg_2SO_4.7H_20$) 2 mg). For our treatments a dilution of 1:5 (DOC = 20 mg/l) was used to simulate the concentration in a river site near the surroundings of an effluent outlet (Carey and Migliaccio 2009, Grantham et al. 2012).

Water parameters such as pH, conductivity (µS/cm), total dissolved oxygen (mg/L) and temperature (°C) were monitored in alternated days (N=19) throughout the experiment using a Multiparameter Probe 3430 WTW.

The experiment had a total duration of 38 days comprising 14 days for biofilm colonization and 24 days for testing the treatments (exposure to stressors). Within the treatment period, grazers´ growth, respiration and grazing activity were tested after 14 days.

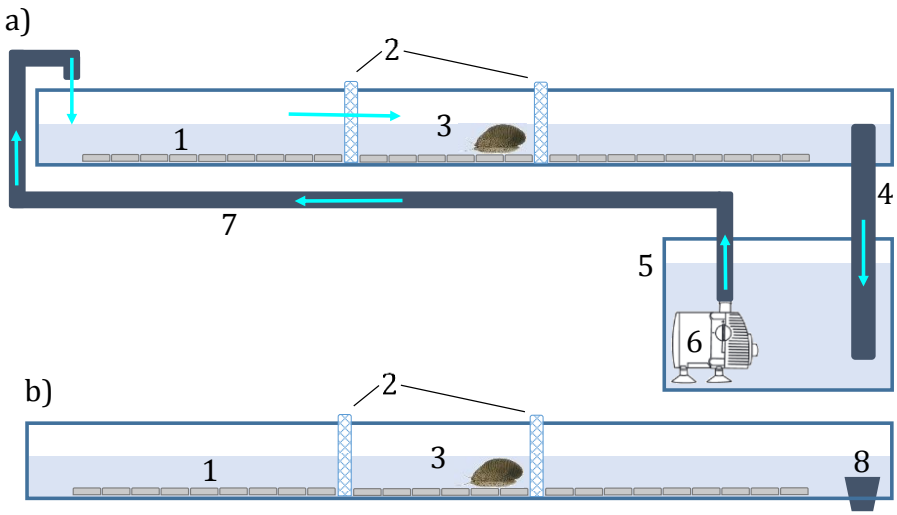

Figure 4.1. Schematic cross-section view of the two experimental mesocosm channels used in the experiment: channels operating in flow-recirculating mode (a) and in non-flow setting (b). 1 - biofilm tiles; 2 - net divisions; 3 - grazing test area; 4 - water depth adjustable outlet; 5 - water reservoir; 6 - water pump; 7 - recirculating pipe system; 8 - water stopper. Arrows indicate water flow direction.

4.2.2. Stressors effects on biofilm

Unglazed ceramic tiles (4 × 5 cm) were placed covering the bottom of the artificial channels and were left to be colonized with biofilm under a basal flow velocity (0.05 m/s) for 14 days. During this period, biofilm inoculum was provided, which was obtained after scraping 6 cobbles from a small local stream (Ribeira do Vale das Flores, central Portugal) and evenly distributed in all channels. Fresh inoculum was provided at days 1 (d1) and 7 (d7). After this initial colonization phase (d14), biofilm was submitted to flow cessation and sewage contamination single and combined effects for 21 days (d21).

At the end of the experiment (d38), biofilm was randomly sampled from each channel to determine the total biofilm biomass (total organic matter expressed as ash-free dry mass (AFDM), mg/cm^2) and algal biomass (chlorophyll concentration; Chl-a, Chl-b, Chl-c, µg/cm^2). Each sample (area = 60 cm^2) was composed of 3 subsamples (3 tiles) which were randomly collected from different positions along the channel to ensure a larger representation of biofilm variability and was taken by scraping the upper surface of the tiles with a toothbrush and washed with distilled water into a flask. Afterwards, each sample was repeatedly centrifuged (3×10 min, 3000rpm), discarding the supernatant until obtaining the correspondent biofilm pellet.

For AFDM determination, biofilm pellets were oven-dried at 70°C for 48h, weighed to get the dry mass, combusted at 450°C for 4h and reweighed to get mineral matter. AFDM was calculated by subtracting mineral mass from dry mass and expressed in milligram per square centimeter.

Chlorophyll determination procedure was adapted from Branco et al. 2010, by extracting the pigments from biofilm pellets with 4 mL of acetone (90%v) in the dark at 4°C for 24 h. To break the cells, samples were macerated and vortexed in a refrigerated environment. After extraction, samples were stirred and then centrifuged (3000 rpm, 10 min at 4°C). Chlorophylls a, b, and c were quantified spectrophotometrically, and their concentration (µg/cm^2) calculated following the procedure of Jeffrey and Humphrey (1975). Chlorophyll a was used as indicator of total biofilm primary production, Chl-c was used as indicator of diatoms amount, and Chl-b was a representation of the biomass from other taxonomic algal groups such as Chlorophyta and Euglenophyta.

4.2.3. Stressors' effects on grazers and grazing activity

Within the mesocosm channels, gastropod (*Theodoxus fluviatilis*) grazers` biomass growth and respiration rate and their grazing activity rate were tested under the different treatments after 14 days (from d24 to d38). This gastropod, widely distributed across Europe, feeds on biofilm by scraping the substrate (Tachet et al. 2010).

T. fluviatilis specimens were collected from a stream site (Rio Anços, central Portugal), transported to the laboratory in aerated containers, acclimatized to the channels' environment conditions 48 h before the grazing test period, and kept fasting for 24 h *prior* to the test. A total of 8 individuals with an initial shell length ranging from 4 to 6 mm were placed in the delimited grazing test area within the channels. This testing area allowed exposure to experimental conditions while restricting grazers to biofilm within that part, enabling further discrimination between biofilm tiles exposed to grazing activity (inside grazing test area) from biofilm tiles non-exposed to grazers (outside grazing test area – control). The density of individuals per channel was chosen based on field observations and on a previous study (Graça et al. 2012).

Grazer's growth (mg AFDM/day) was estimated by assessing the difference between initial and final total body mass for each treatment. *Theodoxus fluviatilis* biomass was estimated using the exponential model $B = 0.17e^{0.5361(L)}$, where B is the biomass (expressed in mg AFDM) and L is shell length (mm) (Graça et al. 2012). Shell length (mm) was measured in each individual (N=8) before and after the grazing test with a caliper and converted into biomass accordingly.

Grazers' oxygen consumption rate (mg O_2/mg ind/h), used as a surrogate of their metabolism, was also determined immediately after the grazing test for each treatment, by employing the Winkler method according Lawton and Richards (1970), with an incubation time of 3h and using 3 individuals per chamber.

For determination of biofilm-grazer interaction, the consumption rate of biofilm by *T. fluviatilis* (mg AFDM/mg individual/day) was measured by the difference between biofilm biomass exposed to grazers and non-exposed biofilm (control biofilm sampled outside grazing test area) considering the biomass of individuals.

4.2.4. Data analysis

Significant differences ($p<0.05$) on biofilm and grazers´ responses for each factor and their interaction were tested using permutational multivariate analysis of variance (PERMANOVA; Anderson and Robinson 2001) with unrestricted permutations of raw data (9999 randomisations). PERMANOVA was run for the two fixed factors "flow velocity" and "sewage" with two levels each (F/nF and S/nS, respectively), based on an Euclidean distance measure. Pairwise post hoc tests were performed whenever an overall effect was significant.

Results for the interaction of flow stagnation and sewage input allowed the identification of the interactions that were additive and those that differed from additive (i.e. significant interaction; antagonism or synergism; Piggott et al. 2015). The expected response if the two stressors had an additive effect (i.e. cumulative effect of single stressors without interaction) was also determined for each biological response. Thus, the predicted response in a scenario of water scarcity and organic load was calculated from the response in a control situation (F+nS), by flow stagnation alone (nF+nS), or an input of sewage alone (F+S) assuming no interaction between factors: Predicted nF+S = F+nS – [(nF+nS) – (F+nS)] + [(F+S) – (F+nS)].

All statistical tests were performed using PRIMER 6 & PERMANOVA+ software (PRIMER-E Lda, Plymouth UK) (Anderson and Robinson 2001).

4.3. Results

4.3.1. Experimental conditions - physical and chemical parameters

Overall, parameters monitored during the experiment (Table 4.1) remained relatively constant among different treatments throughout the trial: mean DO (mg/L) ranged from 9.5 ± 0.1 SE in

control to 11.9 ± 0.3 SE in the combined effect of stagnant flow and sewage treatment; mean conductivity (µS/cm) ranged from 506.2 ± 4.6 SE in the effect of sewage and 576.7 ± 5.9 SE in the effect of stagnant water; pH (mean ± SE) presented the lower variation between treatments ranging from 8.3 ± 0.0 in the control to 8.9 ± 0.1 in the combined effect of stressors, while mean water temperature (°C) varied between 18.1 ± 0.3 SE and 19.5 ± 0.3 SE corresponding to stagnant water single effect and sewage single effect, respectively. The mean DO high concentration under stressor combination corresponds to the increase of photosynthesis activity promoted by the massive amount of algal biomass observed.

Table 4.1. Physical-chemical parameters (mean ± SE) measured in the 14 mesocosm channels during the experiment for each treatment combination. F+nS – control; nF+nS – stagnant water effect; F+S – sewage effect; nF+S – combined effect of stagnant water and sewage.

Parameters	F+nS	nF+nS	F+S	nF+S
Dissolved oxygen (mg/L)	9.5 ± 0.1	9.7 ± 0.1	9.6 ± 0.1	11.9 ± 0.3
Conductivity (µS/cm)	567.5 ± 4.4	576.7 ± 5.9	506.2 ± 4.9	534.9 ± 5.5
pH	8.3 ± 0.0	8.3± 0.0	8.5 ± 0.0	8.9 ± 0.1
Temperature (°C)	19.2 ± 0.3	18.1 ± 0.3	19.5 ± 0.3	18.2 ± 0.3

4.3.2. Effects on biofilm total biomass – AFDM

Results given by PERMANOVA testing the two stressors (Table 4.2) showed that changes in biofilm total biomass were significantly caused by the single effect of flow velocity and sewage contamination, as well as their interacting effect ($p<0.05$). The exposure to sewage single effect (S) increased total biofilm AFDM when comparing with the control (nS) (PERMANOVA pairwise, t= 9.90, p= 0.0001), while the effect of flow stagnation alone (nF) induced the opposite response decreasing total biomass when compared with the control (F) (PERMANOVA pairwise, t= 5.16, p= 0.0004).

A significant interaction of flow velocity × sewage contamination was found for total biofilm biomass using PERMANOVA with the two fixed factors (Table 4.2). PERMANOVA pair-wise comparisons for this interaction showed that biomass within flow stagnation (nF) significantly increased with sewage input (S) when compared with control (nS) (t= 9.41, p(MC)= 0.0287), while within sewage treatments (S), it decreased with flow stagnation (nF) (t= 5.71, p(MC)= 0.0018)

In Fig 4.2, with biofilm biomass obtained for the different treatment combinations, the effect of stressors' interaction (nF+S) revealed a clear trend for higher biomass comparing with single effect of flow stagnation (nF+nS), but lower relative to the sewage single effect (F+S). Total

biomass observed in our experiment under stressors' interaction (nF+S) is lower than biomass estimated based on single stressors additive effect (Predicted nF+S) (Fig. 4.2) indicating an antagonistic (Ant) effect between factors.

Table 4.2. Effects of single and interacting factors of flow velocity (nF; F) and sewage (nS; S) on biofilm biomass, chlorophylls a, b and c concentrations, grazers´ mass increase, grazers´ oxygen consumption and biofilm consumption rate. (PERMANOVA, 9999 unrestricted permutations of raw data based on Euclidean distance measure). Significant p-values (p<0.05) are in bold.

Source of variance	Flow velocity	Sewage	Flow velocity × Sewage
	(*Pseudo-F*) p-value	(*Pseudo-F*) p-value	(*Pseudo-F*) p-value
Biofilm total biomass	(26.628) **0.0006**	(98.02) **0.0001**	(19.271) **0.0026**
Chlorophyll - a	(9.047) **0.0139**	(27.361) **0.0007**	(3.073) 0.1124
Chlorophyll - b	(12.573) **0.0085**	(17.143) **0.0034**	(6.715) **0.0286**
Chlorophyll - c	(39.103) **0.0002**	(19.262) **0.0026**	(0.138) 0.7073
Grazers´ mass increase	(1.279) 0.2774	(0.794) 0.4006	(0.141) 0.7077
Grazers´ oxygen consumption	(4.516) **0.0382**	(3.230) 0.0779	(1.821) 0.1869
Grazers´ biofilm consumption rate	(15.143) **0.0029**	(14.139) **0.0049**	(7.949) **0.0189**

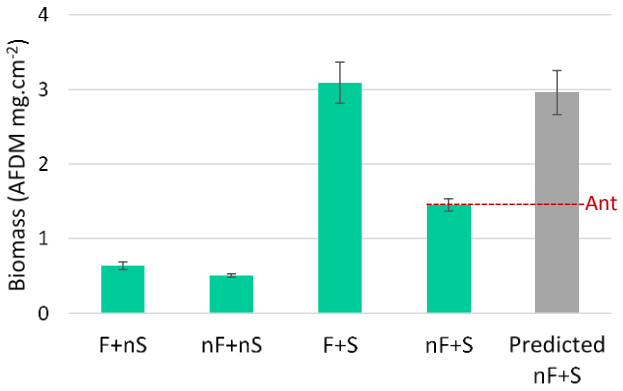

Figure 4.2. Biofilm total biomass (mean ± SE) in each treatment after the experiment (d38). The grey bar indicates the estimated response to stagnant water and sewage treatment given by the sum of individual effects for factors nF and S relative to control (Predicted nF+S). Interaction type between combined stressors in relation to the predicted additive effect is shown (antagonistic; Ant). F+nS – control; nF+nS – stagnant water effect; F+S – sewage effect; nF+S – combined effect of stagnant water and sewage.

4.3.3. Effects on algal biomass – chlorophyll concentration

Algal component of biofilm given by chlorophyll-a (Chl-a) concentration showed significant changes caused by flow stagnation and sewage single effects (Table 4.2).

Chl-a concentration decreased when exposed to flow stagnation (nF) (PERMANOVA pairwise, t=3.01; p=0.0135) and increased when exposed to sewage (S) (PERMANOVA pairwise, t=5.23; p=0.0007) relative to control (F and nS, respectively). Even though the absolute value of the observed Chl-a contents exposed to interacting factors (nF+S) is lower than the Predicted nF+S (Fig. 4.3a), the interaction was non-significant (Table 4.2), possibly indicating additive effects between factors.

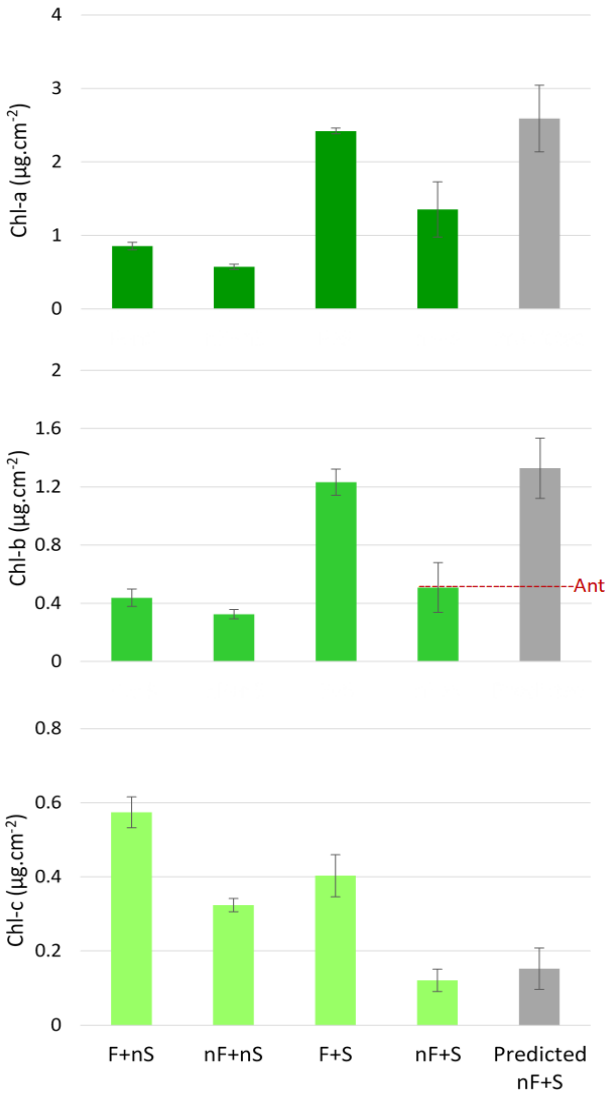

Figure 4.3. Biofilm chlorophyll-a (Chl-a) (a), chlorophyll-b (Chl-b) (b) and chlorophyll-c (Chl-c) (c) concentrations (µg.cm^{-2}; mean ± SE) of the algal assemblages in each treatment after the experiment (d38). The grey bars indicate the predicted response to stagnant water and sewage treatment, assuming no interaction between factors (Predicted nF+S). Significant interaction type between combined stressors in relation to the predicted additive effect is shown (antagonistic; Ant). F+nS – control; nF+nS – stagnant water effect; F+S – sewage effect; nF+S – combined effect of stagnant water and sewage.

Chlorophyll-b concentration responded to single and interacting factors (Table 4.2). Sewage single effect (S) induced a significantly higher concentration for this pigment (PERMANOVA pairwise, t=4.14, p=0.0033), whereas flow stagnation (nF) resulted in a decrease (PERMANOVA pairwise, t=3.55, p=0.0094) of Chl-b comparing with the respective control (nS and F). Regarding the interaction effect, Chl-b within flow stagnation (nF) did not change with sewage input (S) when compared with control (nS) (PERMANOVA pairwise, t=0.89, p(MC)>0.05), while within sewage treatments (S), it decreased with flow stagnation (nF) (PERMANOVA pairwise, t=3.74, p(MC)=0.0091). Moreover, the observed Chl-b found in our experiment under interacting factors (nF+S) is lower than the predicted, indicating an antagonistic effect for the stressors interaction (Fig. 4.3b).

Biofilm chlorophyll c (Chl-c) content responded to both single stressors, but no statistical significance was found for their interaction (Table 4.2), indicating an additive effect between factors. Nevertheless, contrary to the previous algal pigments, Chl-c concentrations decreased after the exposure to single sewage effect (S) relative to nS (PERMANOVA pairwise t=4.39, p(MC)=0.0032), as well as single flow stagnation (nF) relative to F (PERMANOVA pairwise t=6.25, p(MC)=0.0003). In Fig. 4.3c, an obvious tendency for lower Chl-c concentration under the combination of stressors treatments (nF + S) when compared with single stressors treatments (nF+nS and F+S) is visible.

4.3.4. Stressors effects on grazers – body mass increase and respiration

Grazers´ growth measured as body mass increase did not vary significantly under the influence of single, nor combined flow stagnation and sewage factors (Table 4.2). Nevertheless, there is a visible trend for a higher body mass increase under the single effect of sewage treatment (F+S; Fig.4.4a). As for stressors combination, since no significant interaction was found (Table 4.2), their effect on organisms' growth is possibly additive.

Regarding organisms' respiration rate measured as oxygen consumption, an effect to flow stagnation exposure alone (Table 4.2) was found; i.e., grazers presented lower oxygen consumption after exposure to nF when compared with F (PERMANOVA pairwise, t=2.16, p=0.0391). Stressors´ interaction did not differ significantly (Table 4.2), indicating possible additive effects between both factors. A higher respiration rate after sewage single effect (F+S treatments) is perceptible (Fig. 4.4b).

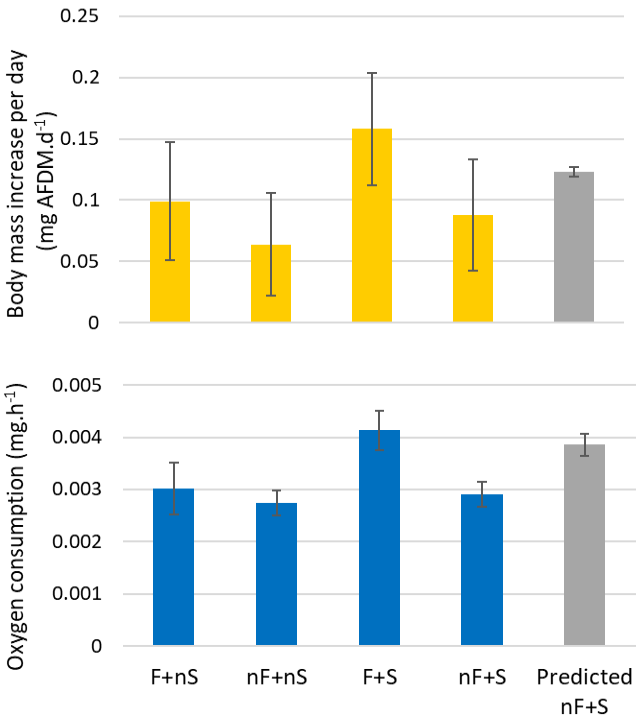

Figure 4.4. Grazers body mass increased rate expressed in mg of AFDM per day (a) and oxygen consumption rate expressed in mg per hour (b) (mean ± SE) measured immediately after the exposure to stressors for 14 days. The grey bars indicate the estimated response to stagnant water and sewage treatment, assuming no interaction between factors (Predicted nF+S). F+nS – control; nF+nS – stagnant water effect; F+S – sewage effect; nF+S – combined effect of stagnant water and sewage.

4.3.5. Stressors effects on biofilm-grazer relation – biofilm consumption rate

Biofilm consumption rate by the grazers was significantly affected by the single effect of stressors and their interaction (Table 4.2). Whereas the stagnant flow alone (nF) decreased biofilm AFDM consumed relative with control (F) (PERMANOVA pairwise, t=3.89, p=0.0029), sewage single effect (S) increased it (PERMANOVA pair-wise, t=3.76, p=0.005) when compared to control (nS). PERMANOVA pair-wise comparisons for the significant interaction between factors that was found in biofilm biomass consumed by the grazers, showed that biofilm biomass consumed by the grazers within nF treatments did not change with sewage input (t=0.92, p(MC)>0.05), while within sewage treatments (S) consumption rate decreased with flow stagnation (nF) (t=4.01, p(MC)=0.0076).

Figure 4.5 shows that grazers´ biofilm consumption rate under the effect of combined stressors (nF+S) was not very different from consumption under the effect of flow stagnation (nF+ nS) but displayed a clear lower value when compared with the single effect of sewage (F+S).

The observed consumption rate for combined stressors (nF+S) was lower than the estimated based on single factors additive effect (Predicted nF+S) (Fig. 4.5), indicating an antagonistic effect for stressor combination.

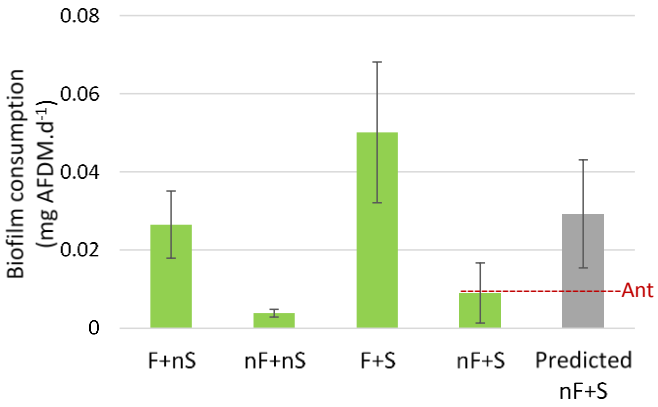

Figure 4.5. Biofilm consumption rate (mean ± SE) expressed in mg of AFDM per mg of grazer per day. The grey bar indicates the estimated response to stagnant water and sewage treatment, assuming no interaction between factors (Predicted nF+S). Interaction type between combined stressors in relation to the predicted additive effect is shown (antagonistic; Ant). F+nS – control; nF+nS – stagnant water effect; F+S – sewage effect; nF+S – combined effect of stagnant water and sewage.

4.4. Discussion

Effluent contamination and water scarcity have been highlighted as two common stressors with major incidence in Mediterranean streams that affect the composition, structure, and functioning of primary and secondary producers in these ecosystems (Grantham et al. 2012, Lu et al. 2016, Neif et al. 2017, Skoulikidis et al. 2011, Timoner et al. 2012). Accordingly, in our experiment, sewage input and flow stagnation showed overall significant single and interacting effects on biofilm and invertebrate grazers. In general, our initial predictions were confirmed and while sewage contamination alone stimulated biological responses, flow stagnation had the opposing effect by diminishing them. Combination of stressors interacted additively or antagonistically, depending on the type of biological response variables considered. For the detected antagonistic interactions, the effect of sewage was clearly reduced by the presence of flow stagnation (antagonist factor) since they acted in different directions. As predicted, flow stagnation weakened the effect of sewage contamination on biofilm biomass growth and also amplified a negative response on grazers' feeding activity and on algae (especially diatoms, Chl-c). If on the one hand, this type of antagonistic cumulative response is recurrent among freshwater stressors' interactions and was previously found with most prevalence at community

and organism level in riverine ecosystems (Jackson et al. 2016); on the other hand, additive effects have also been often registered (Gieswein et al. 2017).

4.4.1. Biofilm biomass (total AFDM and chlorophylls)

Stream biofilms respond to sudden changes in the aquatic environment such as nutrient increases and fluctuations in flow velocity (Romaní et al. 2004, Timoner et al. 2012). Accordingly, our study revealed an increased biofilm biomass under sewage treatments, but a decreased biomass under flow stagnation, as predicted. Whereas organic and nutrient contamination has been related with effects in biofilm mass increase (Matthaei et al. 2010, Lu et al. 2016), water scarcity conditions have shown decreasing biofilm biomass and thickness after desiccation (Corcoll et al. 2015, Timoner et al. 2012) and under slow current velocities (McIntire 1966). Generally, flow increases biofilm settlement and metabolism up to a certain shear-stress level when abrasion starts to remove biofilms´ upper layers (Ponsatí et al. 2016). Furthermore, mass transport by convective flow also maintains nutrient circulation and supply to microbial communities, while water stagnation increases both dead zones and external mass transfer resistance near biofilm surfaces, creating a deficit in such process leading to a decreasing of total biomass. By acting in opposing ways, in this specific case, factors interact antagonistically, i.e., the observed combined response in the presence of both factors was less than predicted additively. According to these findings, one could say that water flow stagnation will limit the biofilm biomass growth (and possible an accentuated eutrophic state) under sewage contaminated environments, at least up to a certain threshold from which an increased water residence time and nutrient retention (Schindler 2006) will overcome that limiting effect.

Moreover, our results indicate that total biofilm AFDM is closely linked to the algal component. Chl-a and Chl-b concentrations have shown a similar response pattern as found for total biofilm AFDM. In addition, one can assume that biofilm total biomass is largely composed by algal communities (Battin et al. 2003, Romaní et al. 2013), which was observed to represent a substantial component in our biofilm. Biofilm Chl-a and Chl-c decreased with flow stagnation and increased with sewage single effect, and although no significant interaction was found between factors for Chl-a, an antagonistic tendency is clearly noticed based on the difference between the observed and the predicted Chl-a concentration. In fact, based on total biomass and Chl-b results and the limiting number of replicates (increasing dispersion) in our design, we assume a high probability of the interaction between flow stagnation and sewage input being also antagonistic. However, looking closer to the main groups of algae present, based on pigment content, we can assume that the significant decrease in chlorophyll-c concentration for

both stressors individually (flow stagnation and sewage) indicate a decrease in the abundance of diatoms, as Chl-c can be used as surrogate for diatom abundance. Also, diatom biomass (i.e. Chl-c) tendentially decreased under the interacting effect of sewage and flow stagnation and probably only the pollutant resistant taxa remained in such conditions (Tornés et al. 2018). These results not only reinforce diatoms' success as ecological quality indicators (Kelly et al. 1998, Stevenson and Pan 1999) detecting changes in flow conditions and pollutant contamination, but also support their potential to detect the cumulative effect of specific stressors such as organic loads and water scarcity (Ponsatí et al. 2016, Tornés et al. 2018).

4.4.2. Grazers' metabolism (oxygen consumption and growth)

Although no statistical significant results were obtained regarding grazers' growth given by their body mass increase during the experiment, a trend for a higher growth under sewage treatment was perceptible. *Theodoxus fluviatilis* has been categorized as a moderately resistant species to pollution according its life traits (Tachet et al. 2010), and such attribute could partly attenuate their response to both single and interacting stressors. Still, growth rates of benthic invertebrates are sensitive to changes in resource quantity (Johnson et al. 2003), thus the tendency for higher biomass of snails observed in our experiment could be a reflection of a higher nutrient uptake though direct feeding on higher biofilm biomass (Cross et al. 2003) found under flow stagnation. Consistent and significant positive effects in the growth of some invertebrates were previously attributed to nutrient-induced changes in the quality of their food resource (Cross et al. 2005). In accordance to this, we found a tendency for lower body mass under flow stagnation single effect, which presented the lower biofilm biomass, meaning less food resource availability and possibly also poorer nutritional quality.

In fact, when looking for organisms´ respiration rates, only flow stagnation influenced grazers´ oxygen consumption resulting in a lower respiration rate. On the one hand, this could be a strategy to cope with adverse environmental conditions (e.g. higher temperatures, lower dissolved oxygen, less food available), but on the other hand it could be a simple reflection of the lower body mass of organisms (Salin et al. 2015).

Nevertheless, given the non-significance of grazers' responses, we acknowledge the need for longer-term studies to corroborate the tendencies observed here. For instance, longer grazing periods (i.e. 25 days) under slower flows were previously found significant in decreasing biofilm biomass (Battin et al. 2003).

4.4.3. Biofilm consumption rate – stressors' impacts on food quality

Biofilm constitutes a key base of the food web, so its abundance and structure may have implications for higher trophic levels (Lamberti 1996). In our study, *T. fluviatilis* seems to feed unselectively on biofilm, since grazing marks type "bulldozer" (Sommer 1999) were observed in the biofilm substrates. Biofilm consumption rates showed an effect for both single and combined stressors. Once more, whereas flow stagnant water had and inhibitory effect on grazing activity, sewage effect was translated in a stimulation of biofilm consumption. These results corroborate our previous findings for grazers' growth and respiration, indicating that grazing activity seems to be influenced by the quantity of food available (i.e. higher biofilm biomass), which in turn is determined by sewage inputs (i.e. nutrient/organic enrichment). Yet again, when stressors acted together, flow stagnation limited the effect of sewage lessening the grazing activity more than the expected based on single stressors cumulative opposing effect.

4.4.4. Conclusions

In this study, flow stagnation has an overall inhibitory effect, while sewage input reveals an increase of biological responses. Our findings suggest that sewage contamination holds a direct effect on the functional response of primary producers and an indirect effect on primary consumers.

When co-occurring, these stressors acted independently (additive effect) or resulted in an antagonistic effect. We assume that for additive interaction, management actions focused on a single stressor should cause a positive outcome, whereas in ecosystems affected by antagonistic stressor interactions, both stressors may need to be removed or moderated to promote any substantial ecological recovery. As supported by our main findings, in a multiple-stressor context, point source pollution (i.e. sewage contamination) often represents the dominant stressor, which should be mitigated before other stressors, because when a single stressor dominates, it is expected that the biological response may reach a threshold past which it will not decrease even in the presence of a second stressor.

Ultimately, we conclude that the effects of characteristic multiple stressor scenarios on Mediterranean stream primary and secondary producers are dependent on water scarcity conditions. Moreover, reductions on sewage inputs need to be given priority by stream managers to improve the resilience of biotic communities and trophic web-based processes to multiple-stressors.

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Chapter 5

Shifts in biofilms´ composition induced by flow stagnation, sewage contamination and grazing

Research article published in *Ecological Indicators*

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5. Shifts in biofilms´ composition induced by flow stagnation, sewage contamination and grazing

Abstract

Freshwaters are constantly facing ecosystem functioning alterations and loss of biodiversity driven by multiple anthropogenic and natural stressors that by acting simultaneously create complex interactions affecting the quantity and quality of water resources.

Stream biofilms are complex communities, which are exposed to these alterations and, in addition, are naturally stressed by invertebrate grazing. Therefore, they are expected to reflect these impacts through shifts in community structure, composition and function. Here we used a mesocosm system to assess the single and interacting effect of major anthropogenic stressors acting in Mediterranean streams (i.e. flow stagnation and sewage contamination) in combination with a biological natural stressor (i.e. grazing) on the main assemblages composing biofilm (i.e. bacteria, fungi and algae) by assessing communities' relative abundance through Denaturing Gradient Gel Electrophoresis (DGGE) operational taxonomic units (OTUs). Biofilm was submitted to the three stressors, in a full-factorial design (2 flow conditions × 2 contamination conditions × 2 grazing settings) in a 5-week experiment. Molecular data showed that the combined effect of anthropogenic stressors (flow stagnation and sewage contamination) induced unequal OTUs responses on biofilm assemblages, with antagonistic effects for bacteria, synergistic for fungi and additive for algae. Sewage and grazing interaction were significant for all groups revealing a negative effect (antagonistic) on bacteria and algae diversity but positive on fungi diversity (synergistic). The same overall response pattern was also found for the triple co-occurring stressors, which increased fungi diversity while decreasing algae and bacteria.

In stream ecosystems in which low flow conditions and sewage contamination prevail in the presence of natural herbivory, algae and bacterial diversity may be severely affected, while fungal diversity may be surprisingly enhanced. Consequently, shifts in the relative proportions could led to the unbalancing of ecosystem processes (e.g., photosynthesis, nutrient mineralization) defined by the microbial communities.

Keywords: Biofilm, DGGE, multiple-stressors, water scarcity, effluents, mesocosm

5.1. Introduction

Freshwater ecosystems worldwide are subject to multiple anthropogenic stressors acting simultaneously and threatening freshwater biodiversity and ecological processes (Jackson et al. 2016, Ormerod et al. 2010). The combination of such diverse stressors on biological and functional responses of stream biota often result in interactions that leads to unexpected ecosystem behaviour with synergisms (amplified effects) or antagonisms (reduced effects) (Folt et al. 1999, Piggott et al. 2015). Those represent a relevant lack of knowledge that poses difficulties and challenges for environmental management and ecological restoration (Birk 2019). Particularly, in arid and semi-arid regions, such as the Mediterranean, water scarcity along with aquatic pollution resulting from effluent discharges and other sources, represent two major co-occurrent pressures that strongly decrease water quality (Navarro-Ortega et al. 2014, Vörösmarty et al. 2010). Mediterranean natural hydrological fluctuation includes contraction periods (Lake 2003) with the reduction of water flow that often leads to stagnant water zones (Gasith and Resh 1999). This decrease in water quantity, worsened by the growing human water demand and climate vulnerability that particularly affect this region (Barceló and Sabater 2010, Navarro-Ortega et al. 2014), will increase water quality deterioration by favouring the impact of point-source pollutants (Petrovic et al. 2011, Ponsatí et al. 2016) and thus, compromising biota diversity and functions. Also, sewage contamination and effluent discharges from wastewater treatment plants can embody a large portion of the total stream discharge, either in temporary or low-flow rivers (Carey and Migliaccio 2009), contributing with high organic loads and elevated levels of nutrients (Smith and Schindler 2009) and other contaminants (e.g. detergents, metals, pharmaceuticals) (Gibson and Meyer 2007, Huerta et al. 2016), that may promote changes in fish and invertebrate community composition (Brown et al. 2011, Grantham et al. 2012) as well as alter the diversity of microbial biofilms (Wakelin et al. 2008). Biofilm microbial community are particularly effective in detecting these environmental changes (Sabater et al. 2007).

As a complex assemblage of diverse communities such as algae, bacteria, fungi and protozoa, biofilms constitute the basis of the trophic chain in streams and are responsible for major riverine microbial functioning and ecosystem processes, including stream metabolism and primary production (Battin et al. 2003, Bechtold et al. 2012, Burns and Ryder 2001, Romaní et al. 2004). Major photosynthesis processes, energy and biomass resources is carried out by primary producers such as algae and cyanobacteria, while biofilm-associated fungi, bacteria and other heterotrophic decomposers contribute significantly to organic matter processing and mineralization of essential nutrients (Pereda et al. 2019, Ruggiero et al. 2006). Consequently, it is expected that biofilms respond differently to a given pressure depending on their core

composition. Algae, bacteria and fungi can be affected in different manners by a mix of different contaminations (e.g. Artigas et al. 2013, Duarte et al 2009). In fact, the composition of biofilm is habitat-specific (Battin et al. 2016), so variations in the structure of biofilms shaped by microbial assemblages under the effect of sewage contamination and water scarcity may significantly influence their functions leading to changes in biogeochemical processes dynamics (Pereda et al. 2019, Ruggiero et al. 2006) and primary production, compromising their nutritional quality as food resource for primary consumers affecting higher trophic levels (Feminella and Hawkins 1995, Liess and Kahlert 2007). Therefore, the first step to understand biofilm functional changes under primary anthropogenic stressors (flow stagnation and sewage contamination), which in turn can be influenced by a natural stressor such as grazing activity, must go through assess structural variations and diversity shifts in biofilm´ main compartments (i.e. algae, fungi and bacteria). Moreover, although many studies have been used aquatic fungi to detect environmental impacts (e.g. Duarte et al. 2017, Ferreira and Chauvet 2011), fungi assemblages within stream epilithic biofilms are often disregard in the assessment of stressors impacts (e.g. Barranguet et al. 2003, Corcoll et al. 2015). As far as we know, fungi assemblages have not been considered when testing the single and interacting effects of water scarcity and sewage contamination under grazing pressures, as part of a stream biofilm.

Given the complexity of undertaking the taxonomic identification and counting of elements of three different taxonomic groups of microorganisms (bacterial, fungi and algae), the use of molecular techniques, such as Denaturing Gradient Gel Electrophoresis (DGGE) has been useful to study microbial diversity of environmental samples and to monitor changes in microbial communities under several environmental pressures (e.g. Corcoll et al. 2015, Duarte et al. 2007, Henriques et al. 2015, Wakelin et al. 2008).

In this study we propose to assess biofilm responses to multiple-stressors, by investigating the single and combined effects of water flow stagnation (physical stressor), sewage contamination (chemical stressor) and grazing activity (biological stressor) on the diversity of biofilm associated bacteria, fungi and algae. As these deploy different functions in the ecosystem, alterations to their natural proportions will be reflected in ecosystem functioning. Specifically, we aimed to assess how biofilm microbial communities' proportions will change under the different stressors combinations, by assessing communities' relative abundance through DGGE operational taxonomic units (OTUs).

5.2. Methods

5.2.1. Experimental design

The experiment was performed in a mesocosm system that consisted in a set of 12 modular independent polymethyl methacrylate channels (150 cm × 10 cm × 12 cm), each one connected to a water tank (27 L) equipped with a water pump (EHEIM compact 3000, Germany), from which water was recirculated (full details *in* Elias et al. 2017). The system was set in an acclimatized room under controlled conditions of temperature (16.8 ± 3.7 ºC, mean ± SD) and photoperiod (12h light: 12h dark). Mesocosms were supplied with 25 L of dechlorinated tap water previously filtered by 5-µm wound polypropylene and active carbon filters, and the water within the channels was maintained at 6 cm depth. Each channel included a grazing test area (28 cm × 10 cm × 12 cm) bordered by 0.2 cm mesh, restricting biofilm consumption activity to that sector.

Unglazed ceramic tiles (4 × 5 cm) were placed at the bottom of the channels to facilitate biofilm colonization under a basal flow velocity (0.05 m/s) for 14 days. Within this period, fresh biofilm inoculum was evenly distributed to mesocosm channels twice (at day 1 and day 7). Biofilm inoculum was obtained after scraping 6 cobbles from a small local stream (Ribeira do Vale das Flores, central Portugal). After this initial biofilm colonization phase (14d), biofilm tiles were exposed to the different treatments for 24 days.

To assess biofilm microbial diversity changes to single and combined effects of water flow stagnation (hereinafter referred as No Flow), sewage contamination and grazing activity we manipulated 3 factors with 2 levels each (i.e. absence of stress vs. presence of stress) in a full-factorial replicated (n = 3) design (Fig. 5.1). Flow velocity included a control basal flow (0.05 m/s) and No Flow (NF; 0.00 m/s, mimicking summer pool conditions), sewage contamination separated non-sewage input (control) from Sewage input (S; proxy of effluent discharge), and grazing activity included non-grazing (control) and Grazing (G; biofilm consumption by gastropods). This design resulted in 8 different treatments that represented a control situation (C), the single effect of each stressor (NF; S; G) and the different stressors combinations: with 2 stressors (NF+S; S+G; NF+G) and 3 stressors combined (NF+S+G), representing the multiple stressors effects (Fig. 5.1).

Sewage input consisted in a 1:5 dilution of a synthetic sewage (OECD 2001; per litter of tap water: peptone 160 mg; meat extract 110 mg; urea (CH_4N_2O) 30 mg; anhydrous dipotassium hydrogen phosphate (K_2HPO_4) 28 mg; sodium chloride (NaCl) 7 mg; calcium chloride dehydrate ($CaCl_2.2H_2O$) 4 mg; magnesium sulphate heptahydrate ($Mg_2SO_4.7H_20$) 2 mg) at a final DOC

concentration of 20 mg/L, simulating a possible sewage quantity found in stream water with effluent contamination (e.g. Carey and Migliaccio 2009, Grantham et al. 2012).

Grazing activity consisted in the exposure of biofilm within the delimited testing area of each channel to gastropod grazers (the snail *Theodoxus fluviatilis*) at a density of 8 individuals per channel (333 ind/m^2). Grazers´ density was chosen based on a previous study (Graça et al. 2012) simulating *T. fluviatilis* natural occurrence. The organisms were collected from a stream site (Rio Anços, central Portugal), transported to the laboratory in aerated containers and acclimatized to the mesocosm channels environment conditions 48 h before the experiment.

Mesocosm water parameters such as pH, conductivity (µS/cm), total dissolved oxygen (mg/L) and temperature (°C) were monitored using a Multiparameter Probe 3430 WTW, every two days (*n* = 19) at same time during the light photoperiod throughout the experiment.

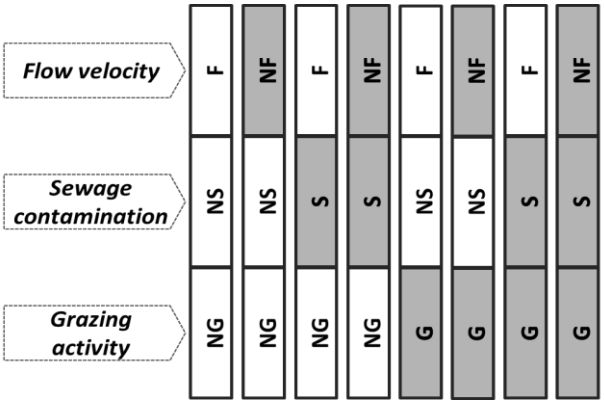


Figure 5.1. Diagram of the experimental factorial design randomly applied in the mesocosm channels, showing the combinations of the 3 factors (Flow velocity, Sewage contamination and Grazing activity) with control conditions (without shading: F – basal flow; NS – no sewage; NG – no grazing) and stressor conditions (grey-shaded: NF – no flow; S – sewage; G – grazing).

5.2.2. Biofilm sampling

After exposure to stressors (at day 24), each mesocosm channel´ biofilm was collected into a 15 mL vial by scraping 3 random tiles (total area ≈ 60 cm^2) with a sterile brush. Vials with biofilm were then centrifuge for 10 min at 1100 G (7516 rpm) in order to remove the excess of water and only a pellet remained. Biofilm pellets were stored at -20 °C until further processing.

5.2.3. DNA extraction and DGGE analysis

Total community DNA was extracted from a homogeneous portion of the biofilm pellets (0.28 g ± 0.04, mean ± SD) using the PowerSoil® DNA Isolation Kit (Mobio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) was used to amplified DNA genes from each biofilm community (i.e., Bacterial 16S, Fungi and Eukarya 18S) using universal primers for each group. Eukarya 18S was used as surrogate of the biofilm´s algal portion. The V3 region of bacterial 16S rDNA was amplified with the primer pair 338F-GC and 518R, the ITS2 region of fungal rDNA was amplified with the primer pair ITS1F-GC and ITS2, and Eukaryotic 18S rDNA gene fragments were amplified with the primer pair Euk1A and Euk516r-GC.

For the three sets of primers, PCR was performed in a final reaction volume of 25 µL containing 6.25 µL NZYTaq 2× Green Master Mix (2.5 mM MgCl2; 200 µM dNTPs; 0.2 U/µL DNA polymerase) (NZYtech, Portugal), 1 µL of each primer, 15.75 µL of ultrapure water and 1 µL of DNA template. Bacterial DNA amplification started with a denaturation step of 5 min at 94°C, followed by 30 cycles of denaturation for 30 sec at 92°C, primer annealing for 30 sec at 55°C and extension for 30 sec at 72°C, followed by a final elongation step of 30 min at 72°C. For fungal DNA, the amplification started with a denaturation of 3 min at 95°C, followed by 35 cycles of denaturation for 30 sec at 94°C, primer annealing for 30 sec at 55°C and extension for 30 sec at 72°C followed by a final elongation step of 30 min at 72°C. For eukaryotic DNA amplification was applied an initial denaturation strep of 3 min at 95°C followed by 35 cycles of denaturation for 30 sec at 94°C, primer annealing for 45 sec at 56°C, and primer extension for 1 min at 72°C, followed by a final elongation step of 30 min at 72°C.

For all PCR experiments, negative controls were used replacing the template DNA with sterile dH2O. PCR reactions were conducted in a Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories, CA, USA). Products were analysed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

Denaturing gradient gel electrophoresis analysis (DGGE) was conducted in a DCode system (Bio-Rad, Hercules, CA, USA). PCR products were loaded onto 8% polyacrylamide gels (37.5:1, acrylamide/bisacrylamide) in 0.5 × TAE buffer (20mM Tris-acetate pH 7.4, 10mM sodium acetate, 0.5 mM Na2EDTA) using a linear denaturing gradient ranging from 35% to 60% for bacteria and from 20% to 50% for both fungi and algae (100% corresponds to 7 M Urea and 40% formamide). Electrophoresis was performed at 60°C, initially for 15 min at 20 V and then for 16 h at 75 V. Afterwards, gels were stained with ethidium bromide (5ug/mL), visualized and captured under UV illumination (Gel Doc, Bio-Rad).

For each DGGE gel was added three lanes (at beginning, middle and end sections) with a standard marker of eight bands for internal and external normalization.

5.2.4. Data analysis

DGGE gels images were aligned and normalized using the BioNumerics 7.6 software (Applied Maths, Sint-Martens-Latem, Belgium). DGGE bands appearing at the same position on the gel were considered as the same operational taxonomic unit (OTU). A matching table containing the relative intensities of each band in bacterial, fungal and eukaryotic fingerprints from each treatment was exported from the software and the average of OTUs relative intensities were used as a proxy of taxon abundance.

Band intensity data from the DGGE gels were square root transformed, and a resemblance matrix was generated using the Bray-Curtis method.

Cluster analysis, with the group average method, was used to group the samples/treatments by similarity.

Significant differences ($p<0.05$) on bacteria, fungi and algae OTUs for each factor and their interaction were tested using permutational multivariate analysis of variance (PERMANOVA: unrestricted permutations of raw data (9999 randomisations; Anderson and Robinson 2001) for the three fixed factors “flow velocity”, “sewage contamination” and “grazing activity” with two levels each (i.e. absence vs. presence of the stressor). Significant results ($p<0.05$) for the interaction of the stressors allowed the identification of synergistic or antagonistic effects (Piggott et al. 2015). The expected OTUs if the stressors had an additive effect (i.e. cumulative effect of single stressors without interaction) were calculated from the response in a control situation (C), by flow stagnation alone (NF), an input of sewage alone (S) or grazing activity alone (G) [example: Expected OTUs NF+S+G = C - (NF + S + G)] for each assemblage whenever a significant interaction between two or more stressors was detected ($p<0.05$, PERMANOVA). Non-additive effects (i.e. cumulative effect of stressor with interaction) were classified as antagonistic (*ant*) if stressors combined effect was less than the expected additively; and synergistic (*syn*) if combined effect was higher than expected additively (Crain et al. 2008).

All statistical tests were performed using PRIMER 6 & PERMANOVA+ software (PRIMER-E Lda, Plymouth UK) (Anderson and Robinson 2001).

5.3. Results

5.3.1. Experimental conditions

Physical-chemical parameters monitored during the experiment were maintained relatively constant for the different treatments (Table 5.1). Mean dissolved oxygen (DO) ranged from 9.5

mg/L in control (C) to 11.9 mg/L in treatments with stressors combined (NF+S). This increase under stressors combination was due to the increase of photosynthesis activity promoted by the massive amount of algal biomass observed. Mean conductivity ranged from 506.2 µS/cm in the S treatment to 576.7 µS/cm in the NF treatment. The mean pH presented the lower variation between treatments ranging from 8.3 in C to 8.9 in NF+S, while mean water temperature varied between 18.1°C and 19.5°C in NF and S treatments, respectively.

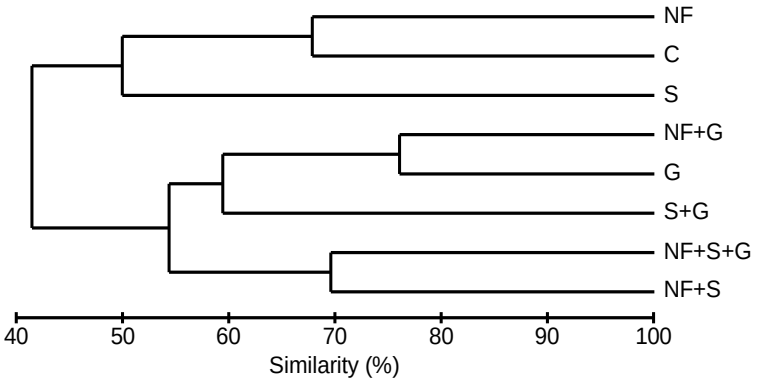
Table 5.1. Physical-chemical parameters (mean ± SE) measured in the 12 mesocosm channels during the experiment (n = 19) for each treatment combination.

Parameters	C	NF	S	NF+S
Dissolved oxygen (mg/L)	9.5 ± 0.1	9.7 ± 0.1	9.6 ± 0.1	11.9 ± 0.3
Conductivity (µS/cm)	567.5 ± 4.4	576.7 ± 5.9	506.2 ± 4.9	534.9 ± 5.5
pH	8.3 ± 0.0	8.3± 0.0	8.5 ± 0.0	8.9 ± 0.1
Temperature (°C)	19.2 ± 0.3	18.1 ± 0.3	19.5 ± 0.3	18.2 ± 0.3

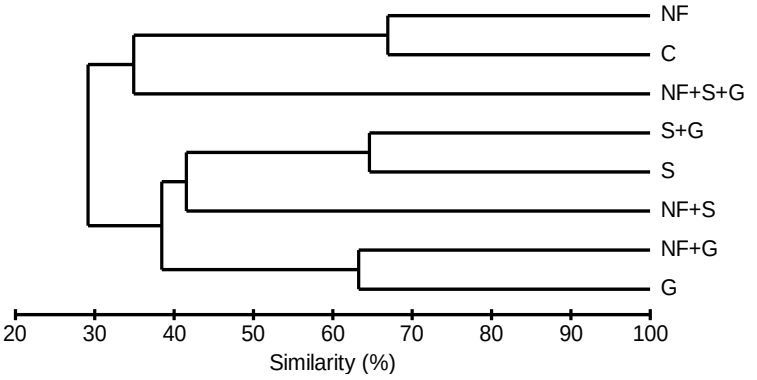
5.3.2. Effects of single and combined stressors on biofilm´ bacteria, fungi and algae communities

Cluster analyses of DGGE fingerprints showed that community structure was overall grouped in two main groups (with approximately 30 to 40% similarity) for each biofilm microbial assemblage (Fig. 5.2). For bacteria and algae, community structure under control (C), and the single effect of Sewage (S) and No Flow (NF) is segregated from community under the influence of Grazing alone (G) and under the influence of more than one factor, i.e. with interacting stressors (Fig. 5.2a, 5.2c). Bacterial DGGE profiles under the single effect of Grazing (G) and the combined effect of Grazing with No Flow (NF+G) presented a high similarity given by cluster analysis (i.e. 75%; Fig. 5.2a). Fungal community under No Flow treatment (NF) yield more than 65% similarity with community of the control situation (C). Despite of community profile under the effect of the 3 factors (NF+S+G) being overall clustered in this group, it presents a lower similarity (Fig. 5.2b) with the remaining (35% similarity). For algae, cluster analysis of DGGE profiles presented a resemblance between community exposed to the interaction of anthropogenic stressors with grazing (NF+S+G) and without grazing activity (NF+S) with similarity values of ca. 85%. Is also noticeable an overall high similarity (i.e. >70%) between communities under grazing activity (G, NF+G and S+G) independently other existing stressors. In fact, the higher similarity for algal community profiles was found between the effect of G and the interaction NF+G (>85% similarity).

a) DGGE OTUs Bacteria



b) DGGE OTUs Fungi



c) DGGE OTUs Algae

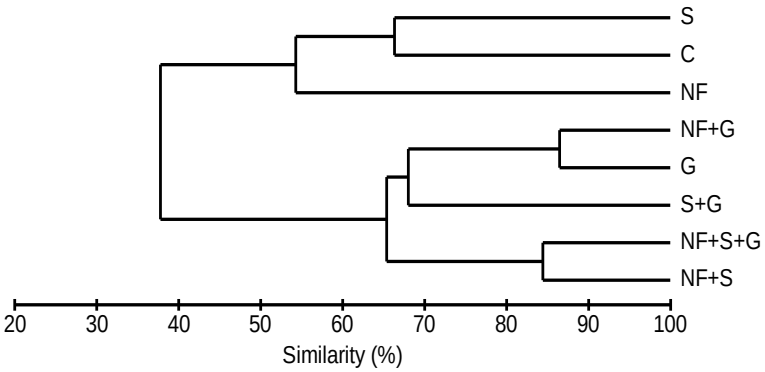


Figure 5.2. Cluster dendrograms of bacterial (a), fungal (b) and algal (c) biofilm communities' profiles (based on Bray-Curtis similarity matrix) assessed from DGGE operational taxonomic units (OTUs) exposed to different treatments testing the single and combined effects of no flow, sewage and grazing activity. C - control; NF - no flow; S - sewage; G - grazing.

Results given by PERMANOVA testing the effect of flow velocity, sewage contamination and grazing activity (Table 5.2) showed significant effects ($p<0.5$) to both S and G and their interaction (S × G) for all biofilm assemblages relative abundances given by DGGE OTUs.

For bacteria, PERMANOVA results showed significant effects for every single and interaction factor (Table 5.2), with a decrease of relative abundance under NF+G treatments and increase for the remaining treatments (Fig. 5.3).

For fungi, our results indicate that community changed significantly under the single effect of S and G, and under the interaction NF × S and S × G (Table 5.2), i.e., whereas S, G and S+G treatments induced a reduction of fungal relative abundance within biofilm, sewage contamination under water flow stagnation (NF+S) lead to an increase (Fig. 5.3).

Regarding algae, significant differences were only detected when testing the single effect of S and G, and their interaction (S × G) (Table 5.2). While algae relative abundances decreased under S treatment, it increased with G single effect. Such stressors in combination (S+G) induced a decreased of algae proportion (Fig. 5.3).

Table 5.2. Effects of no flow velocity (NF), sewage contamination (S) and grazing activity (G) and their interactions on relative abundance of biofilm microbial communities (bacteria, fungi and algae) assessed from DGGE operational taxonomic units (OTUs), given by PERMANOVA (9999 unrestricted permutations of raw data based on Bray-Curtis similarity matrix). Significant p-values ($p<0.05$) are in bold.

Source of variance	Bacteria		Fungi		Algae	
	Pseudo-F	p-value	Pseudo-F	p-value	Pseudo-F	p-value
NF	4.2038	**0.0046**	1.943	0.0869	1.8596	0.1468
S	13.735	**0.0001**	4.9953	**0.0014**	5.4071	**0.0108**
G	13.108	**0.0001**	2.5548	**0.0339**	12.049	**0.0003**
NF × S	7.5514	**0.0002**	4.1261	**0.0044**	2.3737	0.1027
NF × G	4.7778	**0.0019**	1.7876	0.1063	0.12602	0.9765
S × G	6.6904	**0.0003**	6.8874	**0.0003**	4.2798	**0.0216**
NF × S × G	4.272	**0.0041**	0.79019	0.5541	2.2057	0.1161

By using band intensities of each bacterial, fungal and algal DGGE fingerprints from each treatment as a proxy of microbial total abundance (Fig. 5.3), is also visible an overall response to stressors triple interaction (NF+S+G) (although non-significant) by shifting communities' relative abundances more evenly relative to control, and allotting bacteria, fungi and algae in similar proportions within biofilm (Fig. 5.3).

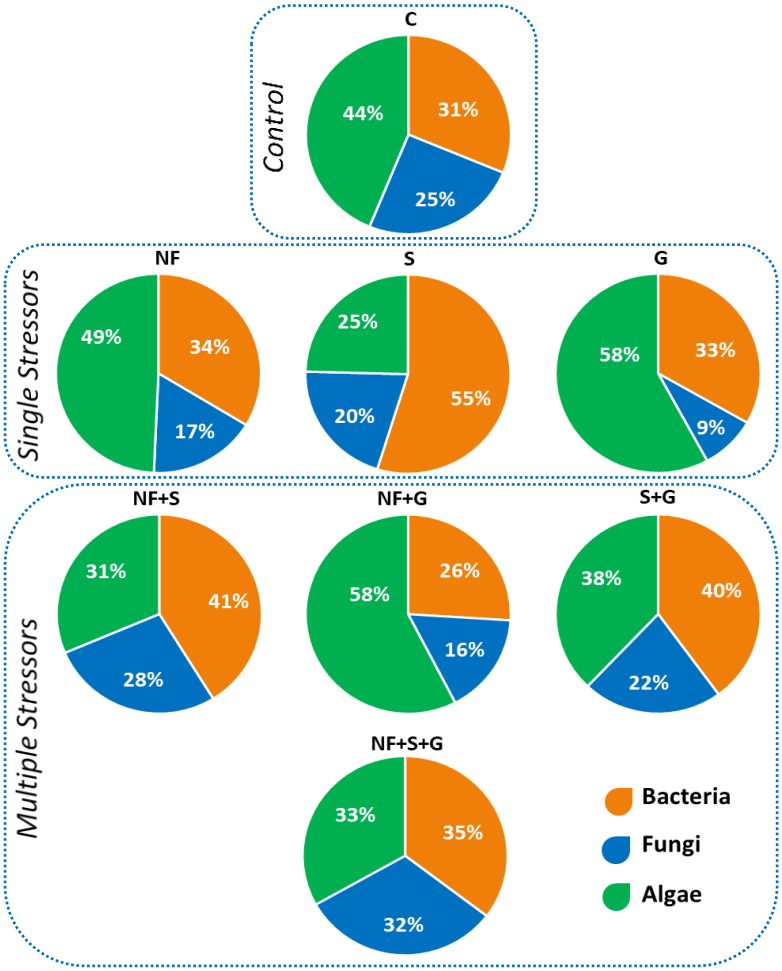


Figure 5.3. Bacteria. fungi and algae proportions (relative abundances) within biofilm submitted to single and combined stressors (mean %) obtained from average of DGGE Operational Taxonomic Units relative intensities. C - control; NF - no flow; S - sewage; G - grazing.

5.3.3. Biofilm diversity changes to single and combined stressors effects – OTUs

In a basal flow velocity condition without sewage contamination or grazing pressure (i.e. control situation without stressors influence), the overall diversity assessed from DGGE operational taxonomic units (OTUs) was higher for bacterial community (17 ± 0.6 bands; mean ± SE), followed by algae (11.3 ± 0.9 bands; mean ± SE) and lastly by fungi presenting the lower diversity (5.3 ± 0.9 bands; mean ± SE) (Fig. 5.4).

Denaturing gradient gel electrophoresis analysis of bacterial communities showed more bands under sewage addition (23.7 ± 3.8; mean ± SE) and less bands under flow stagnation (14 ± 2.1; mean ± SE). Sewage had a clear effect on bacterial OTUs, enhancing diversity in this biofilm compartment (Table 5.2, Fig. 5.4a), while NF induced the opposite response by reducing OTUs

number, when compared to control treatment. When combining the effects of stagnant flow to S effect (NF+S), bacterial diversity was decreased (Table 5.2, Fig. 5.4a).

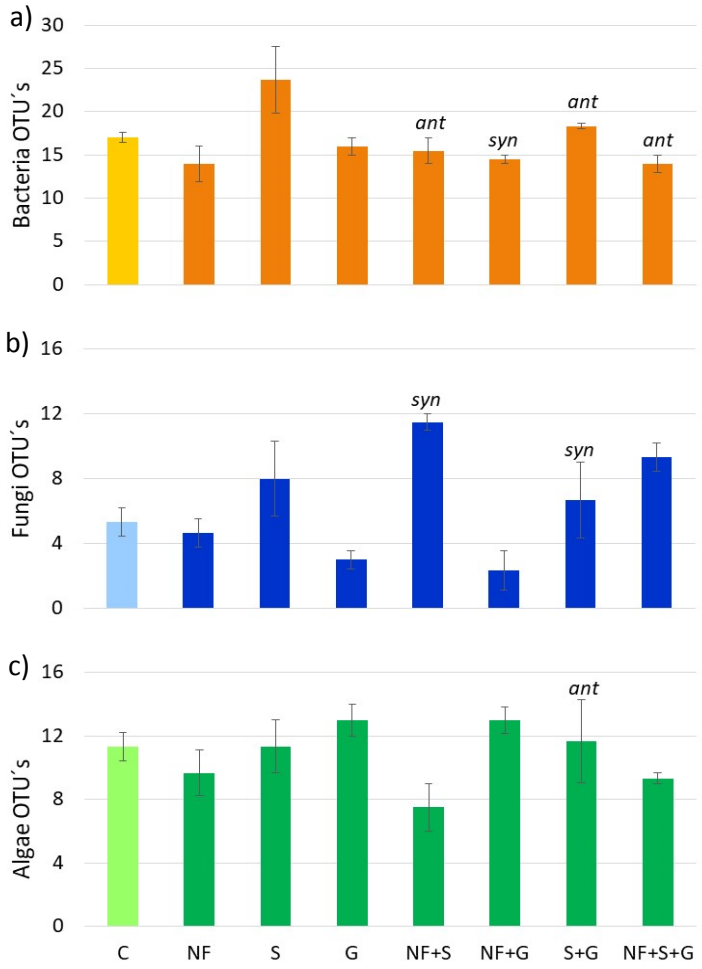


Figure 5.4. DGGE Operational Taxonomic Units (OTU´s) (mean ± SD) for bacterial (a), fungal (b) and algal (c) biofilm communities for the different treatments. Light coloured bars (C) indicate the control treatments (with no stressor). Significant interactions ($p<0.05$, PERMANOVA) are showed as "ant" (antagonism) or "syn" (synergism).

By computing the expected additive effect based on single stressor cumulative response and comparing with the OTUs proportions observed in the experiment, it's possible to determine the interaction effect for each microbial group. Bacterial OTUs observed in this study for the 3 factors interaction (NF+S+G) is lower when compared to the expected OTUs calculated based on the additive sum of individual effects for NF, S and G relative to C, indicating an antagonistic effect (Fig. 5.4a). The same antagonistic effects were found for the double interaction of Sewage with both No Flow and Grazing (NF+S and S+G), while the interaction of NF and G induced a synergistic response in terms of bacterial OTUs (Fig. 5.4a).

For Fungi, DGGE OTUs varied between 2.3 ± 1.2 for NF+G and 11.5 ± 0.5 for NF+S. Fungi OTUs were visibly reduced by grazing activity single effect (G), while sewage contamination (S) increased OTUs number. In fact, when combining S with No Flow (NF+S) or with Grazing (S+G), the observed OTUs were higher than the predicted based on single stressors cumulative effects, indicating a synergistic effect for these interactions (Fig. 5.4b).

Algae DGGE OTUs ranged from 7.5 ± 1.5 under NF+S to 13 ± 1 in grazing single effect (G), presenting less variation in OTUs between treatments when comparing to the other biofilm microbial assemblages. Looking for Figure 5.4c and taking C as reference is shown an increasing of algal OTUs number under the effect of grazing activity (G) and an overall reduction for the interaction of the anthropogenic stressors (NF+S). Yet, an antagonistic effect was found when combining the biological stressor (G) with S (Table 5.2, Fig. 5.4c), since the observed OTUs for this interaction (S+G) was lower than the expected based on single stressors cumulative effects.

5.4. Discussion

Microbial abundance and diversity of stream biofilms is influenced by anthropogenic physical and chemical stressors (Bondar-Kunze et al. 2016, Corcoll et al. 2015, Sabater et al. 2007, Steinman et al. 2011), but also by natural biological stressors such as herbivory (e. g. Graça et al. 2018, Gresens 1995, Mulholland et al. 1991). By acting together, these stressors often generate different responses of those that are expected based on the single stressors (i.e. non-additive effects; Crain et al. 2008) resulting in interactions with synergistic or antagonistic actions. Accordingly, our main findings match these evidences, indicating an overall effect of sewage contamination under water scarcity conditions in the community structure and diversity of stream biofilms, and such effect also showed to be conditioned by an additional natural stressor (i.e. grazing activity). As expected, it was evident that the response to stressors was dependent of the specific biofilm compartment under study, since the three studied stressors (flow stagnation, sewage contamination and grazing activity) affected differently the structure and diversity of bacteria, fungi and algae. Overall when interacting, stressors effects were antagonistic on bacterial and algal diversity but synergistic on fungal diversity. Accordingly, within non-additive interactions between stressors, antagonistic effects have been previously found to be dominant regarding bacterial biofilm compartment (Romero et al. 2018), while synergistic effects were also found for aquatic fungi under the combined effect of nutrient increase and another stressor (e.g. temperature) (Ferreira and Chauvet 2011). As for algae, nutrient enrichment and flow variations combined effect had been previously found to be

dependent on specific algae groups (Bondar-Kunze et al. 2016). Nevertheless, all assemblages responded significantly to sewage contamination and grazing activity and their interacting effect. Whereas bacterial compartment showed a high variation of community profiles with significant stressors interactions for all stressors, fungi and algae responded mainly to sewage contamination and grazing activity single and combined effects. Thus, from the three biofilm assemblages under study, bacterial group revealed to be the most reactive to environmental stressors. In agreement, many studies have proven the bacterial effectiveness and finer resolution in detecting ecosystem impacts though community structure shifts induced by temperature (Henriques et al. 2015) and hydrology (Sabater et al. 2016). Anthropogenic disturbances (Anger et al. 2010, Washington et al. 2013) such as organic loads and nutrient increases (Drury et al. 2013, Hladilek et al. 2016, Wakelin et al. 2008), heavy metals (Ancion et al. 2010) toxic compounds (Muturi et al. 2017, Proia et al. 2013, Rotter et al. 2013) and pharmaceuticals (Corcoll et al. 2015) also cause community structure shifts towards a more impact-related and resistant bacteria (those associated with anthropogenic activities) (Washington et al. 2013).

5.4.1. Single stressors effects

In the presence of a single stressor, all biofilm microbial communities responded mainly to sewage contamination and grazing activity.

Bacteria was affected by all single stressors, with alteration in community structure and increasing their relative abundance, which was specially marked under the effect of sewage.

Accordingly, community shifts induced by wastewater effluents contamination and associated nutrient loads had already been shown (Wakelin et al. 2008). Bacterial relative abundance (based on DGGE fingerprint analysis) increase with sewage contamination, that also increased bacterial number of OTUs, and these findings are consistent with previous works that found higher diversity and increase of specific bacterial abundance in the presence of organic and nutrient water pollution (Hladilek et al. 2016, Naidoo and Olaniran 2014). The additional nutrient and organic supply under sewage input might have fuelled microbial richness and development. On the other hand, some contradicting findings have attributed significant decreases in population size and diversity to wastewater (Drury et al. 2013, Lu and Lu 2014). Drury and co-authors (2013) hypothesize that unfiltered antibiotic substances present in real wastewaters may play a long-term role in the decline of bacteria, which was not the case in our experiment, since antibiotics were absence in our sewage treatments.

Bacterial community also presented more overall diversity (number of OTUs) than fungi or algae, which underlines the inherent (or adapted) high tolerance of this particular biofilm´s assemblage to a broad array of environmental stressors such as temperature, nutrients and several contaminants (e.g. metals, toxicants, pharmaceuticals) (Ager et al. 2010, Hladilek et al. 2016, Wang et al. 2011).

For fungi, flow stagnation overall effect did not cause marked changes in communities' structure, relative abundances or number of OTUs, as one could expect based on previous studies reporting a reduction of diversity and abundance in aquatic fungi associated to low flows (Bruder et al. 2011). In our experiment, the basal flow velocity was rather low (i.e. 0.05 m/s), and the difference with no-flow conditions was not significant enough to be detected by the fungi, but also the biofilm elaborated matrix might have been sufficient fluffy and porous to protect it against water scarcity effects (Battin et al. 2016, Liehr et al. 1994).

Sewage contamination is responsible by organic and nutrient enrichment in aquatic environments (Carey and Migliaccio 2009), which have the potential to stimulate fungal biomass and diversity to a certain threshold (Gulis and Suberkropp 2003, Gulis et al. 2006, Pascoal and Cássio 2004). That could partially explain the observed fungal biofilm OTUs increase under sewage treatments in our study.

Surprisingly, for biofilm algae, and contrasting with evidences (e.g. Smith and Schindler 2009, Woodward et al. 2012) showing boosted algae assemblages under eutrophic environments, our findings indicate that sewage contamination alone did not increase biofilm algal community so promptly as expected, opposing to the evident bacterial and fungal diversity increase. However, compositional changes within biofilms with bacterial and algal increases at different rates and time scales can be induced by the sewage´ nutrient enrichment (Artigas et al. 2013).

The single effect of grazing activity induced an unexpected positive effect on algal biofilm, by increasing both relative abundance and OTUs (based on DGGE fingerprint analysis), which contrasts with most studies that demonstrate the role of herbivory in the declining of periphyton communities (e.g. Guash et al 2006, McCormick et al. 1994, Moulton et al. 2015, Steinman et al. 1992). In our experiment, grazers seem to have potentiated algal abundance and diversity (OTUs), opposing to the decreasing effect observed for both bacteria and fungi with most accentuated effects in the last one. In agreement, invertebrate feeding preference for fungi is, in fact, well documented (Arsuffi and Suberkropp 1989, Gessner et al. 2007, Graça et al. 2001). Thus, we hypothesize that a moderate disturbance induced by grazing organisms in the biofilm mat might have potentiated the establishment and proliferation of new algae OTUs (more intrusive and tolerant), by also creating more micro-niches by grazing and removing the other biofilm assemblages, especially fungi. Additionally, grazing may have removed decaying algal

cells and mitigate the limitation of light within the biofilm mat, benefiting new colonizing stages. Also, this disturbance might have disrupted biofilm matrix realising nutrients that might benefited a higher algal proliferation (Battin et al. 2003, McCormick and Stevenson 1991).

5.4.2. Multiple stressors effects

Multiple stressors interaction was more evident under the combined effect of sewage contamination and grazing activity, as all biofilm microbial assemblages were significantly affected by such condition.

Our findings showed that the combined effect of flow stagnation and sewage contamination induced unequal responses upon the three studied biofilm assemblages. Thus, whereas the combined effect of no flow and sewage was found additive for algae in terms of diversity (OTUs), for the other biofilm assemblages was non-additive in opposing directions, i.e. with antagonistic effects for bacteria and synergistic effects for fungi.

For bacteria OTUs, sewage seem to have mitigated the effect of a second stressor or even a third one, given the antagonistic effect found for interactions whenever sewage contamination was present. This deduction is supported by the synergistic effect found for the interaction of the other two stressors (i.e. No flow x Grazing). Is known that bacterial composition and structure is affected by grazing (e.g. Hahn and Höfle 2001). As hypothesised before, grazing activity might have created more micro-niches by removing other biofilm assemblages and increasing nutrient availability from the matrix (Battin et al. 2003, McCormick and Stevenson 1991) that allowed to contradict the decreasing effect of flow stagnation and resulted in a diversity response above what could be expected based on the single stressors sum effect (Crain et al. 2008).

Under no flow and sewage interaction effect we found a reduction in both algae relative abundance and diversity (OTUs), which is in accordance with previous findings indicating the interaction of pollution and water stress as factor causing the decrease of algal growth (Karaouzas et al. 2018).

Regarding community structure, bacterial, fungal and algal assemblages under the same stressor's interaction (i.e. sewage contamination and flow stagnation effect) presented structural changes, reflecting in part the impact of stressors joint action. Accordingly, previous studies have also detected alterations in community structure of biofilm bacteria, fungi and algae induced by water contaminants and water scarcity effects (Corcoll et al. 2015, Pesce et al. 2016, Proia et al. 2013, Romero et al. 2018).

When addressing the combined effect of sewage contamination with grazing activity, our findings showed that biofilm bacteria and algae followed the same response pattern (antagonistic) that was opposite to fungi (synergistic), meaning that while herbivory natural stressor will worsen the effect of sewage contamination for algae and bacteria, it will create an advantage for fungi diversity increase within the biofilm. Given the synergistic interactions found for no flow × sewage and sewage × grazing for biofilm fungi OTUs, we can assume that sewage contamination made fungal community more sensitive to a second stressor. Similarly, is known that contaminants exposure has the potential to decrease the capacity of periphyton to cope with grazing activity (Guasch et al. 2016). Nevertheless, grazing activity in a biofilm have the potential to favour certain microbial species in detriment of others (Barranguet et al. 2005) increasing the biofilm community adaptation by removing aged less productive biomass and allowing new colonization by tolerant species (Battin et al. 2003), altering biofilm core composition. In fact, multiple stressors effects are capable to select species with higher tolerance, more resistant and opportunistic (Pereda et al 2019, Rotter et al. 2013).

Stream biofilms are naturally exposed to grazing pressures, that in addition to stressors arising from intensive human use, and especially in Mediterranean climate zones, such as flow stagnation and sewage contamination can influence microbial composition in such way that biofilm overall functions in a certain environment may be altered and compromised.

Overall, the co-occurring triple stressors effect increased fungi diversity, while decreased algae and bacteria, following the same pattern observed for the effect of anthropogenic stressors without the herbivory factor (i.e. effect of no flow x sewage). Yet, addressing relative abundance (based on DGGE fingerprints), the triple stressors effect resulted in a shift of the microbial proportions within biofilm distributing them in a more even way, allotting bacteria, fungi and algae relative proportion in similar amounts. In a broader perspective, this may suggest that the effect of these co-occurring stressors has the potential to decrease the natural heterogeneity within stream ecosystems and may contribute to biota homogenization and ultimately, with biodiversity losses.

Given that biofilms are major players in ecological functions in streams, responsible for nutrient recycling, carbon fixation, involved in important ecosystem processes such as primary production (Battin et al. 2016), changing their assemblages' proportions and relative composition may promote alterations in their primary functions, i.e. autotrophic (algae and cyanobacteria) / heterotrophic (bacteria and fungi) ratio can be changed. Thus, variations in the structure and core functions of biofilms produced by microbial communities under the influence

of stressors may affect the aquatic trophic chain and nutrient cycling (Barranguet et al. 2005, Sabater et al. 2007, Woodward et al. 2012).

5.4.3. Conclusions

This study assessed biofilm microbial assemblage's community structure, relative abundance and OTUs diversity (all based on DGGE fingerprints analysis) responses to single and combined stressors effects. Our main findings showed that in a stream biofilm community the same multiple stressor combination can be either unfavourable for some components (e.g. decreased bacteria and algae diversity) and advantageous for another (e.g. increased fungi diversity). Overall, the combination of two main stressors (sewage contamination and water stagnation) common in rivers and streams of the Mediterranean region lead to shifts in biofilms and consequently may interfere in ecosystem functions. In addition, invertebrate grazers seem to be able to restrict the increase of bacteria and algae diversity and might be an important agent in balancing the ecosystem functioning in the presence of those pressures. Therefore, it is important that environmental conditions are also able to support this element of aquatic communities.Biofilms have been used as a reliable tool for bioassessment in freshwater systems (Burns and Ryder 2001, Sabater et al. 2007), but our results suggest that attention should also be given to fungal component of the biofilm as multiple stressors indicator.

Regarding the management of aquatic Mediterranean systems under the impact of water scarcity, our overall results highlight the importance of a strategy based on an integrated river approach to effluent permits allocation combined with effluent reuse and resources recovery technologies. Indeed, even an intensification of wastewater treatment performance, especially during seasonal low flow discharges, may not be enough.

5.5. Acknowledgements

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SECTION IV

MULTIPLE-STRESSORS IN THE “REAL WORLD”

Chapter 6

Effects of variations in water quantity and quality in structure and functions of invertebrates' community of an urban stream

Research article published in *Urban Ecosystems*

Serra SRQ, Calapez AR, Simões NE, Marques JAAS, Laranjo M, Feio MJ (2019). Effects of variations in water quantity and quality in structure and functions of invertebrates' community of a Mediterranean urban stream. Urban Ecosyst 22, 1173–1186, DOI: 0.1007/s11252-019-00892-4

6. Effects of variations in water quantity and quality in structure and functions of invertebrates' community of a Mediterranean urban stream

Abstract

Urban streams provide important ecosystem services to cities' population, from the maintenance of urban biodiversity, temperature, humidity and air quality to improving aesthetics and provision of natural areas for recreation. However, these streams are under multiple-stressors, including artificialization of the channel and flow, poor water quality and cut of riparian vegetation which puts in risk their ecological integrity and consequently their services. In this study, we aimed to understand variations in macroinvertebrate communities and in biological condition as a response to flow and water quality, by following a Mediterranean urban stream over 8 months (December–July). With a monthly periodicity, we sampled invertebrate communities and characterized *in situ* water physicochemical parameters. The urban stream studied showed a high variation of environmental factors over time. Invertebrate communities were generally poor, with some Ephemeroptera (*Baetis* sp.) but was dominated by Chironomidae and Oligochaeta that changed over time alongside with environmental conditions. Biological quality based onthe Portuguese Invertebrates Index (IPtIS) varied between poor and bad. Multivariate community patterns (at genus level) showed variations in communities over time, as well as in their biological trait patterns (invertebrates' maximal size, reproduction mode, resistance form, feeding habits and locomotion mode). Periods displaying worse biological quality, less diverse communities and lower functional richness corresponded to peaks of discharge, higher conductivity and hardness while the best quality communities were found under lower nitrate concentrations. Our study points out that the detection of impacts or recovery in urban streams through invertebrate communities might require at least the use of genus level as family level does not detect smoother changes. Communities reflect the complex interplay of environmental variables affecting structural and functional natural patterns and ultimately the biological condition of this urban stream. Rehabilitation measures should carefully consider this complex interplay of variables to enhance quality and ecosystem services.

Keywords: Urbanization, Multiple-stressors, Low-flow, Contamination, Biological quality, Traits

6.1. Introduction

Urban rivers and streams provide a wide array of key ecosystem services to society such as improving cities aesthetics, resilience and resistance to climate changes, enhancing air quality and carbon sequestration, while providing hotspots of urban biodiversity and green natural areas important for leisure purposes (Bennett et al. 2009, Elmqvist et al. 2015, Palmer et al. 2005, Prather et al. 2013). Although there is a current concern over preserving these services (Elmqvist et al. 2015, Solecki and Marcotullio 2013), the increasing urbanisation demand and industrial growth has come at a heavy cost to freshwater systems as they have been severely impaired by a combination of multiple factors (Vörösmarty et al. 2010). Such factors contribute to the lower resilience found in these ecosystems (EEA 2016) and include increased water abstraction for supplying drinking water and industry processes, discharge of urban wastewater and increase in contaminants which lead to changes in water quantity and quality (Paul and Meyer 2001). Also, urbanization impacts are frequently translated into alterations in geomorphology, removal of riparian vegetation, introduction of invasive species and pathogens, and decrease in biotic richness that is often combined with an increase of pollution-tolerant organisms (EEA 2016, Everard and Moggridge 2011, Nakano and Nakamura 2006). The hydrological regime in urban catchments is severely modified as result of the reduced permeability and capacity to store rainfall of the land surface covered by roads and buildings, as well as channel modifications (e.g. culverting, straightening) and loss of connectivity between the rivers and floodplains (Benke 2001, Booth and Henshaw 2001, Konrad and Booth 2005).Thus, urban streams are strongly influenced by stormwaters, often contaminated by urban runoff characterized by high nutrients and pesticides concentrations and the presence of trace pollutants, exhibiting higher temperatures and unstable substrate condition (Dodds 2006, Kaushal et al. 2010,Musolff et al. 2010). Additionally, in some of these streams high flows may be suppressed for flood control and low flows are generally further reduced because of water abstraction, diversions and dikes. This situation can be aggravated by extreme events (such as prolonged droughts or intensive precipitation) (White et al. 2017a), which are typical in the Mediterranean climate area (Gasith and Resh 1999) but have been worsened by climate change, leading to great damages in cities (i.e. floods, spread of vectors of diseases, undesirable odour). High flows affect macroinvertebrate benthic communities directely by eliminating some organisms, and consequent community traits (Marques et al. 2019), that are dragged downstream or killed (Konrad and Booth 2005), and indirectely by redistributing and changing resourses inputs (nutrients, organic particulate organic matter and preys) and decreasing the available habitats by increasing the intensity of bed scour, disturbing sediments and increasing

turbidity. Even the creation of lateral habitats by inundation may be severely limited in the urbanization landscape given the disconnection of streams with their floodplains by the impermealization of surfaces and the construction of stormwater drainage systems and cut of riparian vegetation (Gurnell et al. 2007, Vietz and Hawley 2019). On the other hand, flow reduction and droughts reduce the area of benthic habitat and available feeding resources, reduce dissolved oxygen and increase nutrients concentrations in the water, leading to smaller populations and reduced community diversity (Calapez et al. 2017, 2018, Konrad and Booth 2005, Nakano and Nakamura 2006). In consequence of these multiple-stressors, invertebrate communities of urban streams become impoverished and lose sensitive taxa, leading to a lower functional diversity and richness and consequently alterations in the ecosystem functioning, that further translate in an even poorer resistance and resilience to disturbances (Feio et al. 2015a, Pyne and Poff 2017). In addition, invasive species, that often have a greater resistance to low flow conditions than native species, may become dominant (Larson et al. 2009).

Among invertebrates, Chironomidae (Diptera) is a characteristic group that often dominates within impoverished urban assemblages, either as result of a high stress level (e.g. Sánchez-Morales et al. 2018), or because the communities are naturally poor. In consequence they have been considered highly tolerant taxa and their power as bioindicators has been neglected (Hawkins and Norris 2000, King and Richardson 2002, Rabeni and Wang 2001, Wymer and Cook 2003). Yet, recent studies have shown that, this family includes genus with a wide sensitivity to disturbance and can be useful indicators of water quality, specially in systems where sensitive taxa such as Ephemeroptera, Plecoptera and Trichoptera (EPT) are absent or very reduced (e.g. Carew and Hoffmann 2015, Milošević et al. 2018, Serra et al. 2017, Raunio et al. 2011, Rosa et al. 2014). However, that requires the identification of Chironomids to the genus level which is often avoided as it needs a higher level of taxonomic expertise and a greater effort in laboratory work (e.g. individuals' preparation and observation under a microscope; Andersen et al. 2013). In addition, using the genus taxonomic level for all taxa, including Chironomidae, allows for a broader and more integrative assessment of the ecosystem integrity through a multiple-trait approach which can provide important indications on the functioning of the urban streams ecosystems (Kuzmanovic et al. 2017). This approach has also the advantage of potentially allowing for a follow-up of the recovery process of streams which is usually very disappointing when a high weight is given to more sensitive taxa such as EPT which are often not abundant or even absent from urban streams and can take very long to recover, even after rehabilitation measures (Feio et al. 2015b, Marques et al. 2019, White et al. 2017b).

Restoration of urban rivers is urgent but requires informed decisions to minimize alterations, maximize structural and functional biodiversity of urban stream biota and enhance multiple

services (Bennett et al. 2009). Understanding how the ecosystem structure and functioning of urban streams is affected by the multiple-stressors and which are the most important stressors affecting the communities is thus fundamental to support effective restoration measures and determine the most effective periods of intervention. Therefore, it is crucial to explore interacting patterns given the surprisingly varied nature of urban rivers due to the multiple alterations at different degrees affecting not only the instream habitats but also surrounding areas (e.g. Gurnell et al. 2007).

Mediterranean rivers present a natural decrease in seasonal discharges that will interfere in the dilution capacity of nutrients and contaminants (Petrovic et al. 2011) and the urbanization of these ecosystems is expected to exacerbate these seasonal variations (Cooper et al. 2013). In the present research we studied a Mediterranean urban stream with two distintive meteorological seasons associated with climatic conditions and hidrological variations that might be determinant to unravel patterns of biota distribution and ecological health of the system: winter (December to February) in which rainfall should determine periods of extreme discharges altering dynamics of nutrients and contaminants given the high impermeable areas surrounding urban streams; and summer (June to August), in which the absence of riparian vegetation, higher temperatures and low rainfall, and higher concentration of nutrients would also impose drought related challenges to aquatic communities. From winter to summer, based on seasonal hidrological variations, we are immediately able identify the transition from conditions that favor organisms adapted to lotic environments (e.g. ephemeroptera) from those adapted to lentic environments (e.g. chironomidae and oligochaeta), though the outcome of the interplay of water flow and water quality with other stressors is far less obvious. In view of that, we aimed to: 1) characterize the macroinvertebrate communities and assess the biological quality, flow and water quality of a Mediterranean urban stream over time; 2) relate flow and water quality patterns with those benthic invertebrate community patterns over the studied period; 3) test the use of invertebrates identified at higher levels of taxonomic resolution (including Chironomidae genera, expected to be very abundant in the urban stream) and biological traits as a finer tool to detect changes in the quality of urban streams. We expect invertebrate's community (taxonomic and trait) patterns to vary over time along with the variation of flow and quality of water given the multiple-stressors that affect this stream located in a highly urbanized area. Sensitive invertebrates should be underrepresented in this stream, with the dominance of tolerant groups representing a loss in functional diversity. Moreover, by using Chironomidae, a major component of invertebrate associated to disturbed streams, at higher taxonomic resolutions should unravel biological (taxonomic, trait) patterns which would otherwise appear indistinct.

6.2. Methodology

6.2.1. Study area and sampling

The study was carried out from December 2016 to July 2017, on a small urban stream (Vale das Flores stream) located in the south of Coimbra (the largest city in Central Portugal; >150,000 inhabitants), which runs directly into the Mondego River through a highly urbanized catchment. The stream is 3.1 km long and is partially channelized and underground. The stream channel becomes uncovered for approximately 1 km long before discharging into the main river (Fig. 6.1) where the study was perfomed, and presented natural substrate stabilized with a discontinuous galvanized metallic net installed over 15 years ago. The riparian vegetation is characterized by a mixture of a few typical riparian trees (i.e. *Salix* spp., *Alnus glutinosa*) and the native woody weeds *Rubus* sp. with invasive reeds, shrubs and macrophytes such as the *Arundo donax*, *Cortaderia selloana*, *Tradescantia fluminensis*, *Ailanthus altissima*, *Ipomoea indica*, *Phytolacca Americana*, *Oxalis pes-caprae*.

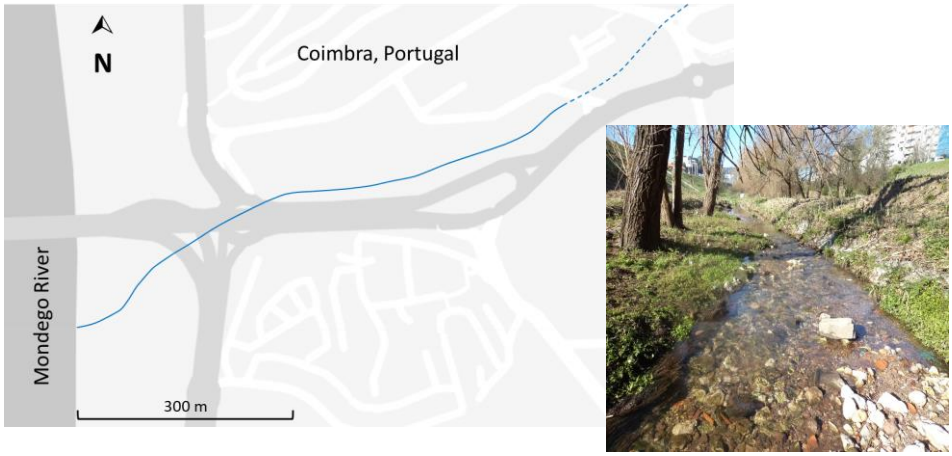

Figure 6.1. Map of the study area. The dashed line corresponds to the channelized underground stream channel and continuous line to the final uncovered stretch (ca. 1 km) that discharges into Mondego river (illustrated by the photo).

Stream discharge (m^3/s) was measured continuously over the study period (every 5 minutes) with a one level gauge (Solinst Model 3001 Levelogger Junior; accuracy 0.1% full scale and a lifetime factory calibration) comprising a datalogger, temperature sensor and pressure transducer that was installed in the bottom of the channel. Additionally, one Barologger (Solinst Model 3001 Barologger Gold) was installed nearby to measure the atmospheric pressure used to barometrically compensate levelogger readings and consequently, obtain the water levels. With the slope, roughness and geometry of the channel, the flow discharge was estimated

through the Manning-Strickler formula computed as $Q = kAR^{2/3}S^{1/2}$, where Q is the volumetric flow rate or discharge (m^3/s), k is the coefficient of Manning-Strickler, A is the cross sectional area of flow (m^2), R is the hydraulic radius (m) and S is the channel slope (m/m). Based on this, a discharge variable related to flow was generated considering the month before sampling (30 days) corresponding to the maximum flow discharge (QMax) which was the most reliable measurement given the type of instrument used. Precipitation (mm) was recorded every minute using a precipitation Pronamic sensor (resolution: 0.2mm) placed on a building rooftop of the Department of Civil Engineering, located near the study stream site (≈ 800 m).

At each sampling occasion, *in situ* environmental parameters of water temperature (°C), pH, dissolved oxygen (mg/L), electrical conductivity (µS/cm) and flow velocity (m/s) were monitored using portable meters (WTW 340i portable multi-parameter probe and VALEPORT 108MKIII flow meter). Water samples were collected for the determination of the concentrations of ammonium (NH_4^+ mg/L; quantification limit (LoQ) of 0.10 $NH4^+$ mg/L), nitrate (NO_3^- mg/L; LoQ of 2.0 NO_3^- mg/L), nitrite (NO_2^- mg/L; LoQ of 0.01 NO_2^- mg/L) and phosphate (P_2O_5 mg/L; LoQ of 0.046 P_2O_5 mg/L) analysed by Continuous Segmented Flow in a Multiparametric Autoanalyzer San++ system from Skalar, with LoQ >0.10 NH4+ mg/L. Total hardness was determined using LCK327 Hach-Lange kits (quantification range: 1 - 20° dH or 5 - 100 Ca^{2+} mg/L and 3 - 50 mg/L Mg^{2+}) in DR 3900 spectrophotometer Hach-Lange according to the manufacturer's instructions. Macroinvertebrate samples were collected in a stream reach (ca. 50 m) in the beginning of every month with a kick-net (0.25 m × 0.25 m opening and 500 µm mesh size), following the official Portuguese multi-habitat protocol covering 6 × 1 m of available habitats (organic and inorganic) proportionally to their presence in the stream reach (INAG 2008). Invertebrates were identified to the highest possible taxonomic level of resolution, mainly species and genus, excluding Oligochaeta and non-Chironomidae Diptera (kept at the family level) and Nemathelmintha, Anostraca and Ostracoda.

Non-Chironomidae invertebrates were identified following Tachet et al. (2010). Chironomidae larvae were mounted on microscopic slides under the stereomicroscope after being digested (KOH, 10%) in a water bath (15–20 min; 85°C), washed in distilled water (5 min) and dehydrated in 70% ethanol (3 min), followed by 96% ethanol (3 min). Heads and bodies were separated and mounted using Euparal medium and identified under the microscope (magnification ×400–1000) to the highest possible taxonomic resolution (mainly genus) following Andersen et al. (2013), Cranston (1982), Lencioni et al. (2007), Prat and Rieradevall (2014) and Rieradevall and Brooks (2001).

6.2.2. Data analysis

Abiotic characterization

A Principal Component Analysis (PCA) was performed on collected environmental data (transformed: Log(x+1)) in order to summarize, in a low-dimensional space, the variance of sampling dates regarding abiotic information.

Invertebrate communities' taxonomic composition

Diversity indices (Shannon-Wiener and Pieolou's evenness) were used to characterize communities. In addition, patterns in macroinvertebrate communities over time were analysed through non-metric multidimensional scaling analysis (NMDS; Bray-Curtis dissimilarity; Log(x+1) transformation) regarding two different levels of taxonomic resolution: 1) at the highest level, mostly genus level except Oligochaeta and Diptera non-Chironomidae (maintained at the family level); 2) at the lowest level considered in regular monitoring programs, mostly family level (except Oligochaeta, maintained at the subclass level). In both analysis Branchiopoda, Ostracoda and Nemathelmintha were counted and considered at the same levels.

Invertebrate communities' trait composition

A set of 10 biological traits with a total of 51 trait categories (Table 6.1) was selected to analyse communities' functional patterns, based on the trait collection of Tachet et al. (2010) database for stream aquatic invertebrates. In addition to this database, trait information of Chironomidae genera (not included in prior database, which only contained subfamily level), was added based on Serra et al. (2016).

Affinities of taxa to each trait category was expressed following a fuzzy ordinal categorization (Chevenet et al. 1994), with the attribution of values from 0 to 5 expressing different degrees of affinity to trait categories, with 0 meaning no affinity, varying from the smallest (1) to the highest (5) possible degree of affinity. Affinities to trait categories were standardized to sum 1 within each trait. Trait-by-samples arrays were computed as the cross-product between the invertebrate abundance and the standardized trait profile.

An NMDS (Bray-Curtis dissimilarity; Log(x+1) transformation) was used to analyse the multivariate functional patterns.

Finally, three multidimensional functional indices were computed and standardized to an interval [0, 1]: Functional Richness (FRic), Functional Evenness (FEve), and Functional Divergence (FDiv) (Laliberté and Legendre 2010, Villéger et al. 2008). These indices were developed to reflect the functional diversity for multiple-traits approaches as an analogy to taxa diversity

components, translating different facets of functional diversity that invertebrate communities contemplate. FRic represents a measure of functional space occupied by the community, FEve describes the evenness of the abundance distribution in functional trait space, whereas FDiv reflects how abundance is distributed with all occupied volume, and divergence of functional characters within the community is maximized if the most abundant species have extreme functional trait values therefore far from the centre of the functional trait range (Mason et al. 2005, Villéger et al. 2008). Theoretically a more functional diverse community is healthier, more resilient and resistant and has higher contribution to the ecosystem functionality (Mason et al. 2005). In urban streams we expect to have a lower FRic, especially in periods with extreme conditions when many organisms with different adaptations are unable to survive (high stress). FEve should be higher when organisms equipped with various traits thrive in more heterogeneous and stable environments; FDiv is expected to be higher in more stable environments where the most abundant species occupy different niches and do not compete (Mouchet et al. 2010).

Former analysis and graphical outputs were obtained using R freeware (R Core Team 2019) and the packages: vegan (Oksanen et al. 2018), FD (Laliberté and Legendre 2010; Laliberté et al. 2014) and ggplot2 (Wickham 2009).

Biological quality

The official Portuguese index for Invertebrates ($IPtI_S$; Ferreira et al. 2008) was applied to the assessment of biological quality over time. The $IPtI_S$ is a multimetric index calculated as:

$IPtI_S$ = Nbr Families × 0.4 + EPT × 0.2+ (IASPT−2) × 0.2 + Log (Sel. EPTCD + 1) × 0.2,

where, EPT is the number of families belonging to Ephemeroptera, Plecoptera, and Trichoptera; IASPT (Iberian Average Score Per Taxon, formerly ASPT') is the IBMWP (Iberian Biological Monitoring Working Party Score; Alba-Tercedor and Sánchez-Ortega 1988) divided by the number of families; EPTCD is the abundance of Chloroperlidae, Nemouridae, Leuctridae, Leptophlebiidae, Ephemerellidae, Philopotamidae, Limnephilidae, Psychomyiidae, Sericostomatidae, Elmidae, Dryopidae, and Athericidae; and Sel. EPTCD is the sum of abundances of EPTCD families. The IPtIS is expressed in Ecology Quality Ratios (EQRs) and a corresponding Ecological Quality Class (High, Good, Moderate, Poor or Bad). All metrics and indexes were determined using AMIIB@ software developed by Instituto da Água, I.P., for the calculation of national indexes based on stream macroinvertebrate communities (available in https://www.apambiente.pt/dqa/amiib@.html).

Table 6.1. Invertebrate biological traits and respective categories used to characterize sampling dates.

Trait designation	Category	Code
Feeding habits	Absorber (absorption through the tegument)	Abs
	Deposit feeder (fine sediment eater)	Dpf
	Shredder	Shr
	Scrapper (grazer)	Scr
	Filter-feeder	Fif
	Predator (cutting or swallowing)	Pre
	Parasite	Par
Locomotion mode, relationship to substrate	Flight	Flr
	Surface swimmer	Ssw
	Swimmer of water column (plancton, necton)	Wsw
	Crawler	Crw
	Burrower (epibenthic)	Bur
	Endobenthic (interstitial)	Int
	Temporary attachment	Tat
Respiration type	Tegument	Teg
	Gills	Gil
	Plastron	Pls
	Spiracle (aerial respiration)	Spi
Potential maximal size	<2.5 mm	S1
	2.5 to 5 mm	S2
	5 to 10 mm	S3
	10 to 20 mm	S4
	20 to 40 mm	S5
	40 to 80 mm	S6
	>80 mm	S7
Resistance form	Eggs, gemmule, statoblast, shell	Eggr
	Cocoons	Cocr
	Housings against desiccation	Hour
	Diapause or dormancy(quiescence)	Diar
	None	Nor
Dispersal mode	Aquatic passive	Aqp
	Aquatic active	Aqa
	Aerial passive	Aep
	Aerial active	Aea
Aquatic stages	Egg	Egg
	Larva	Larva
	Nymph	Nymph

	Adult/imago	Adult
Life cycle duration	Less or equal to 1 year	Lceql1
	More than 1 year	Lcm1
Potential number of generations/year	Less than 1	Less1
	Equal to 1	Eq1
	More than 1	Mor1
Reproduction mode	Ovoviviparity + Care for young	Ovov
	Free isolated eggs	Frie
	Cemented (attached) isolated eggs	Cemie
	Fixed clutches (cemented or attached)	Fixcl
	Free clutches	Frcl
	Clutches in vegetation (endophytic)	Vegcl
	Terrestrial clutches	Tercl
	Asexual	Asex

6.3. Results

6.3.1. Environmental temporal patterns

A set of abiotic variables was used in the characterization of the sampling occasions (Table 6.2). The first-two axes of the PCA performed on all abiotic variables (Fig. 6.2) explain 92.8% of the variance (axis 1: 85.7%; axis 2: 7.1%) of the multivariate scatter sampling dates, segregating mainly December, January, February, March and May from the other months (April, June and July) regarding precipitation patterns. The nutrients concentration (mg/L) varied over the study duration. The poorer water quality periods were found from December to March presenting higher nitrate concentrations and from June to July with high concentration of ammonium and phosphate (Fig. 6.2 and Table 6.2). April, May and February presented the lowest nutrient contamination, especially in terms of ammonium and phosphate concentrations (minimums: 0.57 NH_4^+ mg/L and 0.14 P_2O_5 mg/L in May and February, respectively), whereas the period from April to July present the lowest concentrations of nitrates (7.2 NO_3^- mg/L in June). Dissolved oxygen (mg/L) was lower from May to July (minimum: 3.70 O_2 mg/L in June), period in which water temperature was the highest (maximum: 20.2°C in July); whereas dissolved oxygen was higher in the period from December to March (maximum: 8.33 O_2 mg/L in February), periods with the highest values of maximal daily precipitation (maximum: 24.8 mm in March), hardness

(maximum: 118 $CaCO_3$ mg/L in March) and conductivity (maximum: 380 µS/cm in March). Monthly precipitation was higher in February, March and May, while December, January and April were the months with a higher deviation from mean monthly precipitation, considering both past scenarios, and future simulated scenarios (Fig. 6.3). The period of November to February usually constitutes the period with high precipitation and lower temperatures in Portugal (Fig. 6.3) and is also when there is the high probability of floods or spates occurence. June to August usually represent the drier period of the year (the drier months are usually July and August). The study period was characterized by weak precipitation during December whereas high precipitation occurred mainly in the end of January and beginning of February (when some occasional spates) and with intense but punctual rainfall occurring in May causing only weak spates, followed by periods of low flow in June.

Table 6.2. Abiotic variables measured or determined in each sampling date during the study period (December 2016 to July 2017).

Variable	Sampling date							
	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul
Nitrate (NO_3^- mg/L)	12.0	12.0	12.0	14.0	9.3	9.8	7.2	9.8
Nitrite (NO_2^- mg/L)	0.36	0.62	0.21	0.49	0.36	0.35	0.42	0.52
Ammonium (NH_4^+ mg/L)	0.98	0.93	0.48	0.98	0.73	0.57	4.70	0.66
Phosphate (P_2O_5 mg/L)	0.17	0.63	0.14	0.25	0.19	0.17	0.93	0.37
Hardness ($CaCO_3$ mg/L)	87.3	106.3	107.2	118.9	80.9	66.2	70.0	87.3
Maximum flow discharge (QMax; m^3/s)	0.94	1.59	1.44	0.94	0.51	0.79	1.48	1.59
Water temperature (Temp; °C)	16.6	13.6	14.2	15.0	15.7	15.0	19.1	20.2
pH	7.46	7.01	7.24	7.12	7.23	7.63	7.48	7.63
Dissolved oxygen (DO; O_2 mg/L)	6.70	5.71	8.33	7.29	6.32	5.14	3.70	5.14
Conductivity (Cond; µS/cm)	362	373	333.7	380	253	253	345	362
Flow velocity (FlVel; m/s)	0.497	0.257	0.257	0.257	0.257	0.381	0.132	0.248
Monthly precipitation (Pp_m; mm)	64.0	52.6	77.2	82.0	9.8	73.8	7.6	2.0
Maximum daily precipitation (Pp_dmax; mm)	21.8	19.8	14.4	24.8	9.8	16.8	3.2	0.8

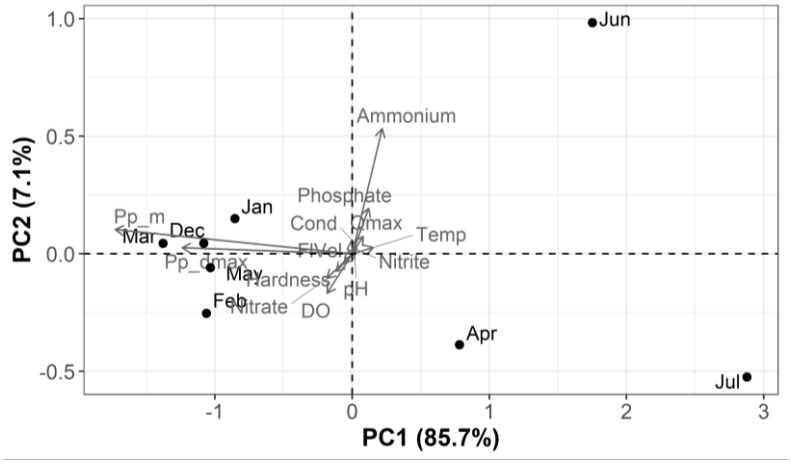

Figure 6.2. First-two axes of Principal Component Analysis PCA projecting sampling dates based on environmental variables from Table 6.2.

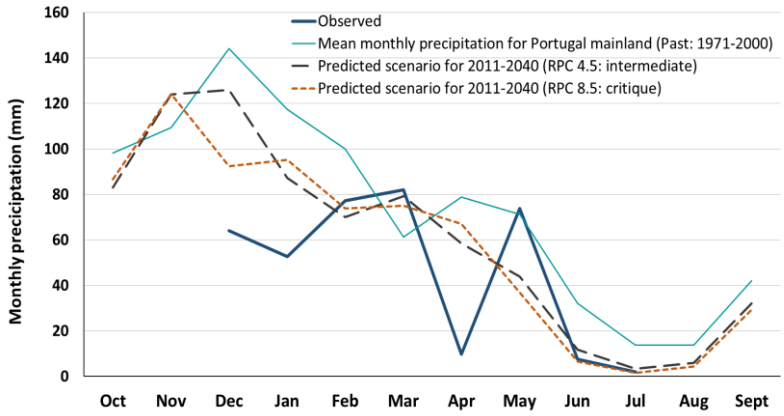

Figure 6.3. Monthly precipitation (mm) compared with historical monthly mean precipitations and considering two selected pathways for climate modelling, simulating future scenarios: intermediate 'Representative Concentration Pathway' (RCP) 4.5; and critique RCP 8.5 of greenhouse gas concentrations (IPCC AR5 2014; Program ADAPT 2015).

6.3.2. Invertebrate community patterns

Macroinvertebrate taxonomic identification of 12 231 individuals showed the presence of 17 families (6 dipteran families, 4 gastropoda families, 4 Oligochaeta families, and one family of Ephemeroptera, Coleoptera and Crustacea - Malacostraca) in the studied urban stream. The most representative taxa were Chironomidae with a total of 7 515 individuals (61%), Oligochaeta with 2 233 individuals (18.3%) and Physidae family (Gastropoda) with 1 708 individuals (14%). The total taxa abundances per month varied from 236 individuals (December) to 2,594 individuals (June). At the highest possible taxonomic resolution, richness ranged from 12 to 27 taxa in December and May, respectively (Table 6.S1 provided as supplementary material).

The ordination of community samples collected over the year, based on highest taxonomic resolution (mainly genus level; Fig. 6.4a) shows a clear segregation of the months of July,

February and December from each other and the remaining months. December is characterized by a low number of taxa (12) and abundance (236) and communities were dominated by Oligochaeta (73%, represented mainly Lumbriculidae and Tubificidae) and Chironomidae (25%, mainly *Orthocladius* sp.) (Fig. 6.4a and Table 6.S1). January was highly dominated by Chironomidae constituting 92% of the total abundance (represented mainly by *Eukiefferiella* sp., *Orthocladius* sp. and *Rheocricotopus* sp.), whereas in February was found a higher abundance of Oligochaeta (59% of the total abundance represented mainly by Lumbriculidae and Tubificidae) followed by Chrinonomidae (35%, represented mainly by *Eukiefferiella* sp.) (Fig. 6.4a and Table 6.S1). The rest of the months were mostly dominated by Chironomidae (representing from 55 to 74% of the total abundance) except for July (with more Oligochaeta representing however only 22% of the total abundance and only few Chironomidae genera (representing only 8% of the total abundance). The segregation of July is the result of a higher abundance of gastropods such as *Potamopyrgus antipodarum* and *Physa fontinalis* (both introduced exotic gastropods), together with the presence of *Procambarus clarkii* (an exotic crustacean), only found during this sampling date (Fig. 6.4a and Table 6.S1).

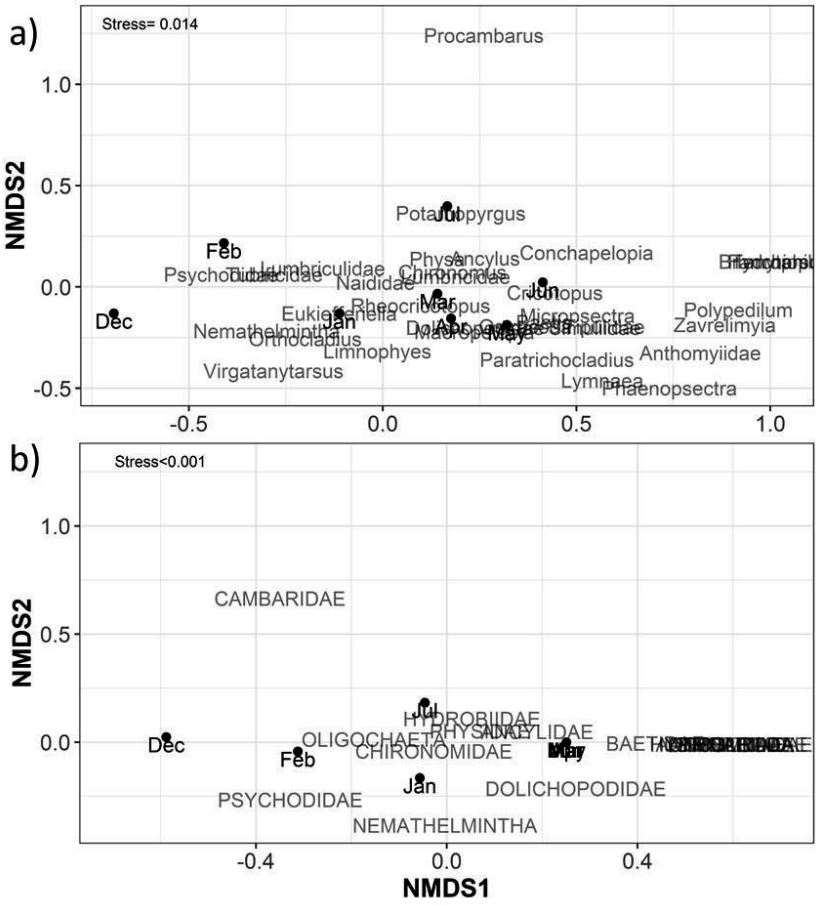

Figure 6.4. Non-metric multidimensional scaling analyses (NMDS) based on taxonomic composition of monthly samples at: a) Genus level; b) Family level; with the superimposition of taxa distribution.

At lower levels of taxonomic resolution (Fig. 6.4b), December and July are also segregated from the remaining, but the differentiation of monthly communities is smaller, and it is not clear which are the taxa responsible for the segregation (except for *Procambarus clarkii*, Cambaridae, in July).

6.3.3. Invertebrate trait patterns

Traits were gathered for 99.9% of total individuals collected along the sampling dates. The traits that presented fewer taxa with available information were dispersal mode and life cycle duration (yet trait information was found for more than 65% of the taxa present). On the other hand, organisms with fewer trait information were the genera *Paratrichocladius* sp. and *Virgatanytarsus* sp. (no information for 5 and 6 traits, respectively). *Paratrichocladius* sp. represents only 0.5±0.9% of the total abundance in sampling dates, never exceeding 2.3% in the month in which they were most abundant (May), whereas *Virgatanytarsus* sp. represent only 0.1±0.2% of the total abundance, never exceeding 0.4% (December).

The NMDS based on invertebrate traits categories (Fig. 6.5) shows a more similar pattern to the one found in genus-based NMDS (Fig. 6.4a), but with a higher similarity between the months of December and February which are better represented by absorbers (Abs) and deposit feeders (Dpf), interstitial and water column swimmers (Int and Wsw respectively), organisms with potential sizes larger than 20 mm (S5, S6, S7), with asexual reproduction (Asex) or with sexual reproduction through eggs in cemented or attached clusters (Fixcl), without resistance forms (Nor) or with resistance through cocoons (Cocr). To a lesser extent these two months are also characterized by taxa smaller than 2.5 mm (S1) and aquatic passive dispersers (Aqp), life cycles lasting more than one year (Lcm1) and more than one generation per year (Mor1) (Fig. 6.5). July is the most segregated month due to higher proportions of ovoviviparous taxa (Ovov, determined mostly by reproduction mode of present Crustacea), crawlers (Crw) and surface swimmers (Ssw), and invertebrates with intermediate sizes (S4, 10 to 20 mm) (Fig. 6.5). In June there is a small differentiation from spring samples based on higher proportions of scrapers (Scr) and invertebrates with aquatic adult phases (Aqp or Aqa), an increase in organisms with diapause or housing as a resistance strategy (Diar or Hour), and also an increase in breathing through gills (Gil) and plastron (Pls) (the last usually not very well represented in other sampling dates) (Fig. 6.5). The remaining months appear close together with slight differences. Traits that were clearly responsible for the segregation of December, February and July included potential maximal size, reproduction, resistance, feeding and locomotion mode.

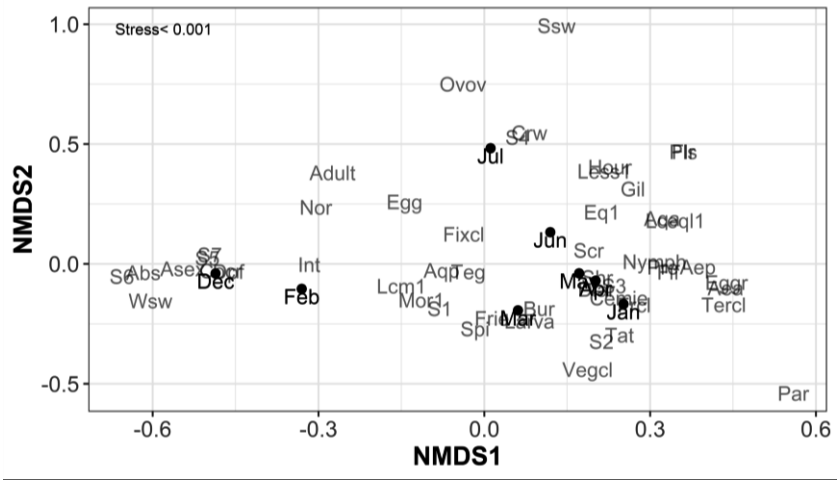

Figure 6.5. Non-metric multidimensional scaling analyses (NMDS) based on traits composition of monthly samples, with the superimposition of trait categories distribution.

The first three sampling months (December to February) are characterized by a low Functional Richness (FRic), the metric attains the highest values in March, May and June (Fig. 6.6a). On the contrary, Functional Evenness and Divergence (FEve and FDiv respectively; Fig. 6.6b, c) fluctuate in the first period of sampling, decreasing and stabilizing in the following periods before increasing again in July. During the sampling dates the maximum FEve values occurred in December and February, with the metric delineating an overall decrease along time; whereas the FDiv maximum was obtained in July, showing an overall increase in the metric along the sampling period.

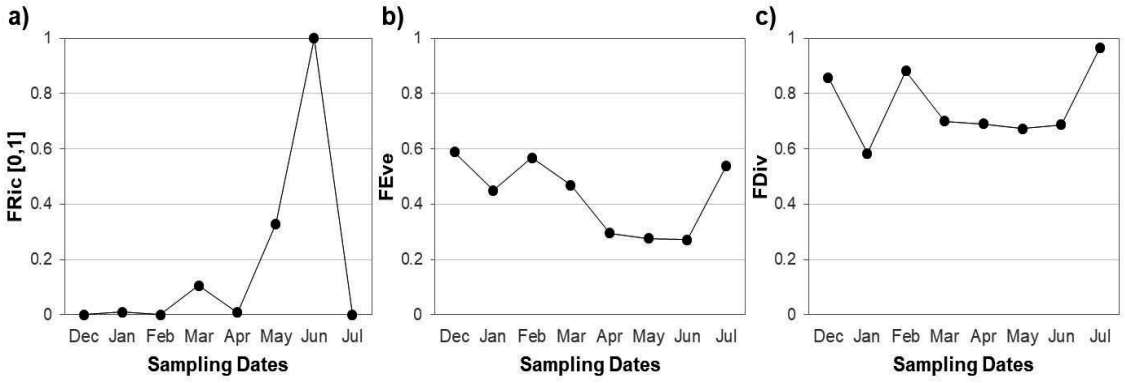

Figure 6.6. Temporal variation in: a) Functional richness; b) Functional evenness; c) Functional Divergence; based on the invertebrate community traits of the urban stream.

6.3.4. Biological quality temporal patterns

The $IPtI_S$ and metrics (Table 6.3) indicate a general low biological quality (varying between poor and bad), which was better in May and June and worse in December and February. This

impoverished quality was accompanied by a low number of families used in $IPtI_S$ (between 4 and 12), particularly Ephemeroptera (represented by a sole family Baetidae and a unique genus *Baetis* sp.) and the absence of Plecoptera and Trichoptera (Fig. 6.4 and Table 6.S1). In spring, from April to June, the biological quality was higher based on the values of IASPT and $IPtI_S$, but summer months have the highest values of diversity and evenness.

Table 6.3. Metrics based on invertebrate communities sampled over the year and $IPtI_S$ scores and classes.

Metrics	Sampling date							
	Dec	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**
Number of taxa identified	12	19	12	21	21	27	25	14
Number of taxa used by the $IPtI_S$	4	9	6	9	9	11	12	7
Total abundance	240	1236	386	2516	1937	2132	2602	1312
IASPT	2.5	3.38	3.17	3.63	3.67	3.64	3.67	3.17
Shannon Wiener diversity	0.66	0.38	0.9	0.83	0.88	1.01	1.25	1.08
Evenness	0.48	0.17	0.5	0.38	0.4	0.42	0.5	0.56
EPT taxa	1	1	0	1	1	1	1	1
EPT individuals	1	1	0	44	139	97	96	1
sel EPTCD	0	0	0	0	0	0	0	0
$IPtI_S$	0.135	0.287	0.189	0.302	0.304	0.343	0.365	0.235
Quality class	Bad	Poor	Bad	Poor	Poor	Poor	Poor	Poor

6.4. Discussion

Streams of urbanized areas suffer from a wide range of perturbations known as the "urban stream syndrome" (Walsh et al. 2005, Wenger et al. 2009). One manifestation of this syndrome is the impoverishment of communities that become dominated by Chironomidae and Oligochaeta with the loss of sensitive taxa (e.g. Feio et al. 2015a, Suren and McMurtrie 2005, Walsh et al. 2001). In agreement, we found that a mixture of different factors is affecting stream community throughout the year, resulting in low richness and abundance of EPT (Ephmeroptera, Plecoptera and Trichoptera), the most sensitive groups of benthic invertebrates. In addition, there was no obvious differentiation in community composition by season (i.e., winter: December-February; spring: March-May; summer: June-July) in spite of existing variations in community and traits composition over the studied period. This is an interesting pattern which is probably a reflex of an atypical precipitation pattern (Fig. 1), with a generally low precipitation along the studied period but at the same time higher values in June than in April, which are

unexpected and could be a consequence of climate changes (IPCC 2012). In addition, it is important to note that the degree of impermeability of the lands surrounding urban streams will condition the runnof of the stream drainage, wich are sensitive to rainfall rate and to the density of drainage networks mangement (Ogden et al. 2011), and these variable features explain the less predictable nature of an urban river in a given time.

Looking into the biological information we can see that May and June were the sampling dates with better biological quality (higher $IPTI_S$ scores, number of EPT individuals and functional richness), that also showed an obvious environmental pattern of lower nitrate concentration. Opposing to these, December, February and July displayed the worse biological quality and associated environmental patterns of higher peaks of discharge (maximum daily flow) and higher values of conductivity and hardness. As previously shown (Ledford et al. 2017, Paul and Meyer 2001), urbanization increases surface runoff due to the impervious surfaces, promote drastic alterations in streams hydrology and water chemistry as result of the transport of nutrients from land to the stream (such as the phosphates). This could influence biological patterns that translated into a low biological quality, like those found in February and March, in spite of the higher concentration of water dissolved oxygen that might favour more sensitive taxa. The higher values of hardness and conductivity found in our study could be a reflex of a higher dissolution of the alkaline substrates (including calcareous rocks) found in the study area, while the higher phosphate concentration is probably a result of soils leaching. The effect of peaks of discharge could be potentially less impacting if the water level was constantly higher, which could be improved by a better management of streams.

The traits-based analyses brought also some additional conclusions. In the worst quality months (December and February) we observed a higher proportion of generalist species, with generalized feeding strategies (absorvers and deposit feeders), and bigger taxa adapted to slower water velocity and living associated to silt and sandy substrate (Chironomidae and Oligochaeta). Also, some of the dominant taxa in the worst quality months have asexual reproduction modes being able to build cocoons and to resist to adverse conditions (e.g. Lumbriculidae e Tubificidae). This is in agreenment with other studies showing that very unstable or disturbed environments disfavour the presence of specialist taxa (Büchi and Vuilleumier 2014, Poff and Allan 1995, Verberk et al. 2010). Functional richness confirmed the previous patterns being higher in May and June. In contrast, FEve and FDiv presented low values for the same period, indicating a low evenness of abundance distribution in community traits. These results show that a reduced number of individuals, in this case more sensitive taxa that appeared in those months, have contributed to the higher functional richness. Recent studies showed also that urbanization is responsible for the homogenization of stream invertebrate trait

diversity (e.g. Barnum et al. 2017, Kuzmanovic et al. 2017) and an overall homogenization of biodiversity (e.g. McKinney 2006).

Considering restoration measures, traits may add a mechanistic perspective of the effects of urbanization, helping the definition of priority measures (e.g. Alberts et al. 2018, Marques et al. 2019). In this case, if the occurrence of generalist taxa, with broadest niche width, increases during periods of low biological quality, resulting in functional homogenisation of communities, the target of restoration measures should be the habitat characteristics that allow different narrow-niche specialist taxa to occur, for example, shredders, scrapers, gill and plastron breathers (Clavel et al. 2011). In addition, it is important to improve water quality, avoiding high levels of nutrients, as this factor combined with low flow affect communities in complex ways (Calapez et al. 2019, Jackson et al. 2016) leading to a worse biological quality.

The presence of various exotic species (*Physa fontinalis, Potamopyrgus antipodarum, Procambarus clarkii*) has also contributed to the segregation of sampling dates, being represented by a higher number of individuals (gastropods and crustaceans) in the last sampling periods, thus contributing with around 3% of the total abundance from December to April, up to 9% in May, to 20% in June and 65% in July. The unnatural flow regime and the homogeneity of habitats charactering urban stream favour the presence of tolerant but also of exotic species, and the dominance of generalist and opportunistic taxa (Booth et al. 2016, Helms et al. 2009, Jones and Leather 2012). Other studies have also shown nitrate concentrations to be positively correlated with the number of exotic species (Vermonden et al. 2009).

The community of macroinvertebrates (including Chironomidae) in Mediterranean streams vary over time reflecting natural changes in water physicochemical characteristics and watershed runoff (e.g. Álvarez-Cabria et al. 2010). Therefore, in an undisturbed stream the structure of the communities tends to be more homogeneous if sampled within the same season of the year and differ to the structure found in another season. In this study, we observed that variation over time did not translate temporal proximity. Although we can not show the existence of cause and effect relationships among obtained patterns, these patterns should be a reflex of a year of atypical precipitation and multiple hydromorphological and water quality stress gradients interacting among each other, that determine the uncertain character of urban streams when compared to more naturalized streams. Multiple interacting stressors determine effects that can be synergistic, antagonistic or additive (Crain et al. 2008), varying over time and depending not just on the stressors and their magnitude but also on their interplay. Communities' responses to these combined effects will vary dramatically given organisms' sensitivities and tolerance, ecological trade-offs and on local adaptation patterns (Sabater et al. 2019).

In our study we were able to detect slight variations in invertebrate communities of the urban stream over time, corresponding to small changes in environmental conditions. Yet, most ecological restorations studies observed no changes in invertebrate communities, leading authors to defend that they are not usefull indicators to monitor streams under rehabilitation or restoration, as a large amount of change is needed before being reflected in the improvement of communities (Palmer et al. 2010). However, we argue that the result might depend on how the community is assessed as most restoration follow up monitoring uses indices at family level (e.g. Selvakumar et al. 2010), relying in the genus or species taxonomic level only for non-Diptera (e.g. Lepori et al. 2005, 2006), identifying Oligochaeta and Chironomidae only to family or subfamily level (e.g. Sundermann et al. 2011) or even excluding Chironomidae diptera from the analyses (e.g. Louhi et al. 2011). In fact, here the higher variations in communities over time are observed at a higher taxonomic resolution (mostly genus level) and corresponding trait diversity, when compared to the quality index $IPtI_S$ which is mostly based on a lower taxonomic resolution (family level). Furthermore, the identification of Chironomidae at a higher resolution allows a better discrimination of the impact of stressors in the community, as this family is highly abundant and ubiquitous and have species and genus with different sensitivities (e.g. Serra et al. 2017; Milošević et al. 2018), being among the first colonizers that shows fast recovery rates after impacting events (Casas and Langton 2008, Čerba et al. 2011, Marziali et al. 2010, Raunio et al. 2011). For example, species of the genus *Conchapelopia* were associated to high/good/moderate stream habitats, whereas species of the genus *Chironomus* were indicator of our samples in different months, and the first is considered sensitive while the second is a tolerant genus (e.g. Calle-Martínez and Casas 2006, Marziali et al. 2010, Orendt 2018). Additionally, Chironomidae may be used as indicators of conditions of interest for restoration, such as rheophilic and thermal conditions (Marziali and Rossaro 2013, Lencioni et al. 2012). Therefore, we defend that Chironomidae genus are a good tool to follow the quality of this type of streams and their ecological recovery after restoration measures, especially in initial steps where other better-known sensitive taxa remain absent (as the EPT).

6.4.1. Conclusions

In conclusion, the results of this study showed that: 1) the invertebrate communities of the urban stream are generally impoverished which is reflected in the biological quality index; 2) the communities varied over time but their patterns do not follow a seasonal pattern as it would be expected under natural conditions; 3) communities reflect the interplay of various stressors (e.g. a higher conductivity and nitrate concentrations together with intensive precipitation and peaks

of discharge; 4) higher variations in communities over time are observed at genus level (including many Chironomidae genera, crustacea and gastropod) and based on multiple biological traits (mostly potential maximal size, reproduction, resistance, feeding and locomotion invertebrates related) than at family level or by the application of the biological quality index $IPtI_S$.

6.5. Acknowledgements

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6.7. Suplementary material

Table 6.S1. Taxa abundance in each sampling date.

Taxa used by the IPtIs	Taxa identified	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul
Ancylidae	*Ancylus fluviatilis*	0	7	5	24	28	33	100	73
Anthomyiidae	Anthomyiidae	0	0	0	0	0	5	1	0
-	Branchiopoda	0	0	0	0	0	0	1	0
Baetidae	*Baetis* sp.	1	1	0	44	139	97	96	1
Cambaridae	*Procambarus clarkii*	0	0	0	0	0	0	0	2
Chironomidae	*Chironomus* sp.	2	9	3	34	73	60	97	76
Chironomidae	*Conchapelopia* sp.	0	1	0	0	3	1	15	4
Chironomidae	*Cricotopus* gr. *bicinctus*	2	0	0	19	16	114	318	8
Chironomidae	*Eukiefferiella* gr. *claripennis*	8	435	129	1593	739	553	62	0
Chironomidae	*Limnophyes* sp.	1	15	0	14	22	10	0	0
Chironomidae	*Macropelopia* sp.	0	0	0	1	1	0	0	0
Chironomidae	*Micropsectra* sp.	0	8	0	19	130	195	695	2
Chironomidae	*Orthocladius* sp.	37	428	2	33	260	168	11	0
Chironomidae	*Paratrichocladius* sp.	0	0	0	8	31	49	0	0
Chironomidae	*Phaenopsectra* sp.	0	0	0	0	0	1	0	0
Chironomidae	*Polypedilum* sp.	0	0	0	0	0	2	5	0
Chironomidae	*Rheocricotopus fuscipes*	8	219	1	83	135	292	216	15
Chironomidae	*Tanytarsus* sp	0	0	0	0	0	0	14	0

Chironomidae	*Virgatanytarsus* sp.	1	2	0	0	0	5	0	0
Chironomidae	*Zavrelimyia* sp.	0	0	0	0	0	1	1	0
Dolichopodidae	Dolichopodidae	0	1	0	0	1	0	1	0
Hydrobiidae	*Potamopyrgus antipodarum*	0	2	2	8	0	2	11	24
Hydrophilidae	*Hydrophilus* sp.	0	0	0	0	0	0	1	0
Lymnaeidae	*Lymnaea* sp.	0	0	0	0	1	2	0	0
Nemathelmintha	Nemathelmintha	0	2	0	1	0	0	0	0
Oligochaeta	Lumbricidae	0	7	3	3	6	23	9	7
Oligochaeta	Lumbriculidae	77	19	145	337	141	213	310	235
Oligochaeta	Naididae	3	13	11	59	34	29	26	10
Oligochaeta	Tubificidae	93	12	68	137	58	32	80	33
-	Ostracoda	0	0	0	3	0	1	0	0
Physidae	*Physa fontinalis*	3	33	11	66	71	192	513	819
Psychodidae	Psychodidae	0	3	3	0	0	1	1	0
Simuliidae	Simuliidae	0	0	0	12	8	9	8	0
Tipulidae	Tipulidae	0	0	0	3	1	3	2	0
Number of identified taxa		12	19	12	21	21	27	25	14
Total abundances		236	1217	383	2501	1898	2093	2594	1309

SECTION V

GENERAL DISCUSSION

Chapter 7

Discussion and Conclusions

7. Discussion and Conclusions

7.1. Research overview and discussion

The subject of this Thesis is grounded on the growing evidence that most freshwater ecosystems are exposed to multiple anthropogenic stressors and natural disturbances in such way that the potential for interactions among them are expected in unexpected ways, undermining biological processes, functions and biodiversity (Hering et al. 2015, Ormerod et al. 2010). Therefore, understanding the impact of multiple stressors is of utmost importance to identify, predict, and prevent riverine ecosystems from further deterioration and to provide helpful insights for restoration practices (Côté et al. 2016).

Aquatic pollution is not only one of the most recurrent pressure of riverine ecosystems, but also consists in a complex threat that embody itself a multitude of stressors (e.g. oxygen depletion, nutrient enrichment, toxicity, higher temperature) given its diverse origin nature and composition. This is of special concern in Mediterranean rivers, which are affected by water scarcity and high flow variability and also represent the major recipients of significant pollutants inputs from diffuse and point sources (Navarro-Ortega et al. 2012, Sabater et al. 2014).

The set of studies composing this Thesis, focused on stressors interactions effects derived from such significant pressures predominantly acting in Mediterranean streams, i.e. water scarcity and water pollution, presenting evidences of such effects at different biota levels over the course of the chapters. A simplified overview of this research work is summarized in Table 7.1, including the methodological approach and the temporal scale used to assess biota responses, the stressors or main variables that were evaluated, the biological community used, the measured responses or indicators of stressors effects and the overall key results.

Mesocosms assays, as the ones described in the present thesis (Sections II and III), mimic simplified ecosystems in which diverse biotic communities (biofilm - algae, bacteria, fungi - and macroinvertebrates) and their responses can be tested in factorial designs tailored to simplify and discriminate between multiple-stressors effects. Such methodological approach also allowed for testing functional changes based on biological traits (Chapter 3) and enabled the inclusion of interaction between biotic communities (i.e. primary producers and consumers; Chapters 4 and 5). Assessing aquatic community's functional response to multiple-stressors and measure their response at different trophic levels enhances the ecological relevance of the assays, yielding a better understanding of how ecosystems respond to particular stressors.

Table 7.1. Summary of research works developed under the scope of multiple-stressors effects by chapter attending to the type of approach used and duration of the assays, the tested stressors or variables assessed, the type of organism used, the indicator or response measured and the overall key results. Main effect of stressors interaction, whenever assessed, are shown in bold.

Chapter	Approach type (temporal scale)	Stressors/variables	Organisms	Indicator/response	Overall stressors effects
2	Outdoor Mesocosm (3 hours × 2 seasons)	Flow reduction (vL) Oxygen depletion (dO)	Macroinvertebrates	Drift	Decreased with vL and increased with dO; stressors interaction was **synergistic**.
3				Biological traits	Stronger shift induced by the effect of single stressors, notably in dispersal and respiration mode.
4	Indoor Mesocosm (3 weeks)	Flow stagnation (NF) Sewage (S)	Macroinvertebrates - Grazers	Respiration rate	Decreased with NF; Stressors interaction was **additive**.
				Growth rate	Non-significantly affected but showed a trend to increase with S.
			Biofilm	Total biomass	Decreased with NF and increased with S; stressors interaction was **antagonistic**.
				Algal biomass	Decreased with NF and increased with sewage; Stressors interaction was **additive**.
			Biofilm/Grazer	Consumption rate	Decreased with NF and increased with S; stressors interaction was **antagonistic**.
5		Flow stagnation (NF) Sewage (S) Grazing (G)	Biofilm´ bacteria	Microbial structure and diversity (OTUs)	Interaction of NF×S was antagonistic; NF×S×G effect was **antagonistic**.
			Biofilm´ fungi		Interaction of NF×S was synergistic; NF×S×G effect was **additive.**
			Biofilm´ algae		Interaction of NF×S was additive; NF×S×G effect was **additive.**
6	Field experiment – case study (8 months)	Discharge peaks; Precipitation; Flow velocity; Seasonal variation; Nutrients; Hardness; pH; Conductivity; Dissolved oxygen	Macroinvertebrates	Abundance and diversity	Overall poor community with low diversity and high abundance of Chironomidae (61%), Oligochaeta (18.3%) and Gastropoda (14%).
				Biological traits	Worst water quality related with higher proportions of individuals with generalist feeding, adapted to low water velocity, preference for silt/sandy habitats and producers of resistance forms.
				Biotic quality indexes	Overall poor/bad biological quality scores; Lowest quality associated to higher peak discharges, higher conductivity and hardness; Better quality related to lower nitrate concentrations.

In this research, stressors were tested using a gradient of experimental manipulation ranging from a mesocosm facility in an acclimatized room with adjustable temperature and photoperiod (Section III), to an outdoor mesocosm system exposed to natural climate conditions (Section II), and finally, to an *in situ* assessment in a case study (Section IV). Also, exposure to stressors effects were measured at different time scales, ranging from 3 hours, (Chapters 2 and 3), to 3 weeks (Chapters 4 and 5), to 8 months (Chapter 6).

Overall, throughout the experimental works carried out in mesocosms systems were tested different levels of hydrological alterations related with water scarcity, from flow velocity reduction (Section II – Chapters 2 and 3) to water flow stagnation (Section III – Chapters 4 and 5) and different water contamination conditions derived from water pollution such as, oxygen depletion (as a consequence of organic loads) (Section II) and sewage inputs (mixture of nutrients and other compounds) (Section III). The overall single and combined effect of these stressors were detected at different levels of important stream communities, which are crucial for ecological functioning in streams (i.e. primary producers – stream biofilm; and primary consumers – macroinvertebrates) by testing several responses of these communities such as drift behaviour (Chapter 2), biological trait alteration (Chapter 3), biomass, metabolism, growth and feeding activity (Chapter 4) and microbial proportion changes (Chapter 5). Also, in Section IV, a case study in a Mediterranean urban stream (Chapter 6), provided support to assess patterns of hydrological and physicochemical alterations over time in a natural multiple-stressor context. In this study, besides testing macroinvertebrate communities` in terms of taxonomic and trait composition over time, alterations in stream biological quality were also evaluated.

Whenever assessed, stressors interactions found thorough this research were predominately additive or antagonist, despite synergistic effects had also been detected (Table 7.1). Such finding is in accordance with previous studies, in which additive stressors effects were the most recognized on aquatic flora and fauna (Gieswein et al. 2017) or antagonism effects had the most prevalence at community and organism level in freshwater ecosystems (Jackson et al. 2016).

In this chapter is presented an overall discussion of the main findings of Chapters 2, 3, 4, 5 and 6, which provides a broad knowledge of multiple-stressors major effects, particularly deriving from water scarcity and water pollution. In addition, some recommendations are inferred to prevent and mitigate degradation of river ecosystems under such pressures and improve its management.

7.1.1. Effects on macroinvertebrate responses (Section II)

Does flow reduction and oxygen depletion combined effects influence macroinvertebrate drift behaviour? And is that response higher than expected based on the knowledge of single stressors effect? Based on our findings in Chapter 2, the overall answer to both these questions are affirmative. Even though the individual stressors low-flow velocity and low dissolved oxygen concentration had opposing effects on invertebrates´ drift behaviour (i.e. by decreasing and increasing it, respectively), their combined effect was higher than the expected based on the observed single effects and thus, stressors interaction induced a positive synergistic drift on the community. As supported by another studies (Brown et al. 2013, Crain et al. 2008, Hanazato and Dodson 1995, Piggott et al. 2015), the positive synergistic effect detected for any pair of levels of the two stressors seems to indicate that one stressor reduces the tolerance to the other stressor. In our study, oxygen depletion conditions changed the effect of flow velocity reduction by shifting its negative effect on drift rates to a positive effect, indeed increasing the drift.

Do seasonal changes determine the drift behaviour to co-occurring stressors effects? Macroinvertebrate drift patterns varied between the two studied seasons (i.e. Winter and Spring) and were found evidences (Chapter 2) that drift response was influenced by the interaction of season and water dissolved oxygen condition. In fact, stressors combined effect in Winter seemed to intensify drift response when hypoxia was higher, while the same effects during Spring were independent of the hypoxia level. These findings may suggest that under water scarcity, macroinvertebrates sensibility to different levels of oxygen depletion during Spring are lower than in Winter, even though the higher mean temperature observed in Spring. In contrast, higher water temperature has the potential to reduce oxygen supply while increasing biological oxygen demand in the system and increasing invertebrate sensitivity to oxygen deficits in the water column (Pardo and Garcia 2016, Winter et al. 1996). Therefore, our differences are most likely linked with seasonal variations in communities' sensitivities and vulnerabilities, which can change temporally during their lifespan and vary between life stages (Dudgeon 1990, Rincón and Lobón-Cerviá 1997, Schreiber 1995), than with temperature.

Are drift responses to stressors interaction influenced by macroinvertebrates community composition? Even though the co-occurrence of low flow and oxygen deficits had induced an amplified drift response (i.e. synergistic) of the community as a whole in Chapter 2, the assessment of individual taxa may generate different results given the preference of some groups for slow flowing waters (i.e. Echinogammarus sp., Proasellus sp., Baetis sp.; Tachet et al.

2010) and the ability to cope with oxygen deficit of others (i.e. Orthocladiinae and Tanitarsini; Tachet et al. 2010). For instance, while a decrease in flow velocity is considered a stressor to rheophilic taxa, it can be expected to enhance the habitat suitability for other limnophilic taxa which prefer slow current velocities, such as the case of Baetis sp., Caenis sp., Echinogammarus sp. and most Diptera (Schmidt-Kloiber and Hering 2015, Tachet et al. 2010) that were found in our experiment (Chapter 2). Also, contrary to the overall community trend, was found that taxa such as Echinogammarus sp., Proasellus sp., Elmis sp. and Simuliini drifted less under the effect of oxygen depletion and this might be related with some physiological adaptations and specific respiratory traits (Flynn and Bush 2008, Tachet et al. 2010).

With these findings in mind, the need to a further assessment considering organisms´ functional responses (i.e. traits) was highlighted and addressed in Chapter 3. By establishing classifications of species based on their attributes rather than their taxonomy has often provided a mechanistic understanding of anthropogenic stressors impacts (Verberk et al. 2013) and thus, is expected to relate species to ecosystem functioning (directly or indirectly; Feio and Dolédec 2012, Webb et al. 2010), making it possible to compare ecosystems and assemblages composed of different species pools (Lamouroux et al. 2002).

Which are the major effects of flow decreasing and oxygen depletion on macroinvertebrate functional composition? Overall, single effects of flow velocity reduction and severe oxygen depletion determined a shift in community traits (Chapter 3). When evaluating the stressors´ combined effect in macroinvertebrate functional composition, no significant interaction was detected at this response level, in opposition to the synergistic interaction found when considering the response at community composition level (i.e. taxon relative abundance) addressed in Chapter 2. Accordingly, there are other evidences that biological trait composition could be less sensitive and can suffer weak changes than taxonomic composition and abundance to specific stressors (e.g. Bêche et al. 2006, Bonada et al. 2007). However, when looking for each individual trait category, it was detected a clear overall effect of the stressors acting in combination.

Which biological traits would be likely to be filtered out in a scenario of increasing hypoxia during a low flow event? Low flow triggered alterations in proportions of specific macroinvertebrate traits in the drift (i.e. respiration and resistance form), with implications in trait diversity losses. This finding is supported by Schriever et al. (2015), which attributed a decreased functional trait diversity to drastic stream flow reductions. Functional trait diversity has significant implications in the capacity of benthic assemblages to withstand and recover

from disturbances and to react to environmental changes (Poff et al. 2006). Specifically, the main findings in Chapter 3 allows to infer that while flow reduction alone will specifically filter out the gill breathers' individuals as well as the ones that use eggs as resistance form in response to adverse conditions, the combined stressors will impact more drastically organism's passive dispersal and locomotion by swimming. Such loss in trait diversity in the macroinvertebrate community can result in variations in ecosystem functioning through, for example, local changes in biomass, secondary production, stream metabolism as well as resulting in biodiversity losses or alterations of its distribution patterns (Cardinale et al. 2012).

The use of some key trait categories would be a valuable contribute to detect specific alterations in the ecosystem while providing an indirect link to ecosystem functioning. For example, organisms that use gills and tegument as respiration mode responded in opposing ways to the single effect of low flow and to the combined effect of low flow with two levels of oxygen depletion, which indicate that the respiration mode of organisms can potentially discriminate flow velocity decreasing among additional hypoxia conditions and can be used as an early warning signal of ecosystem problems.

7.1.2. Responses of stream biofilms and primary consumers (Section III)

In what way do sewage contamination and water flow stagnation affect river biofilms (i.e. primary producers)? Whereas sewage contamination single effect stimulated stream biofilms responses increasing total biomass and chlorophylls concentration, flow stagnation had the opposing effect by diminishing them (Chapter 4). These findings were expected given that organic and nutrient contamination has been implicated in biofilm´ mass increase (Matthaei et al. 2010, Lu et al. 2016), and water scarcity conditions have showed decreasing biofilm biomass after desiccation and under slow current velocities (Corcoll et al. 2015, McIntire 1966, Timoner et al. 2012). Though, stressors combined effect was lesser that the expected based on their single effects, interacting in a non-additive way by inducing an antagonistic response on stream biofilm biomass. So, is possible to infer that water flow stagnation will limit the biofilm biomass growth (and a possible eutrophic state) under sewage contaminated environments at least up to a certain threshold from which the increased water residence time and nutrient retention will overcome that limiting effect (Schindler 2006). Regarding algal component assumed by chlorophylls concentration, an additive effect for stressors combination was detected.

Are stressors effects up-scaled to the following trophic level (i.e. primary consumers) through direct effects on grazers' metabolism and indirect effects over biofilm quality as food resource?

Although the answer to this question was not straightforward, our overall findings in Chapter 4 indicated a tendency for an affirmative answer, suggesting that sewage contamination has a direct effect on the functional response of primary producers (i.e. stream biofilm) and an indirect effect on primary consumers (i.e. grazers), and such effect is particularly influenced by water flow stagnation.

A propensity for higher growth of consumers was noticed under sewage contamination, while under water stagnation effect was found an opposite tendency, indicating a lower body mass. Accordingly, grazers´ oxygen consumption rates decreased significantly with flow stagnation. These findings could be directly related with the quantity and quality of biofilm (i.e. food resource) accessible under such conditions. For example, while under sewage contamination was observed higher biofilm biomass (and overall grazers´ higher metabolism), under flow stagnation biofilm biomass were lower, meaning less food resource availability and possibly also poorer nutritional quality. Consistent and significant positive effects in the growth of some invertebrates were previously attributed to nutrient-induced changes in the quality of their food resource (Cross et al. 2005). The lower oxygen consumption rates of grazers observed under sewage contamination could be a possible strategy to cope with worse ecological conditions (e.g. higher temperatures, lower dissolved oxygen, less available resources), or just a simple physiological consequence of the lower biomass of the organisms (Salin et al. 2015).

Regarding biofilm consumption rates (interaction grazer/biofilm) was found that while stagnant flow decreased biofilm consumption rate, sewage effect increased it. Such finding corroborates our previous results for grazers' metabolism, inferring that grazing activity seems to be influenced by the quantity of food available, which in turn is determined by nutrient and organic enrichment resultant of sewage inputs. The combined stressors effect on grazers´ biofilm consumption rate was found to be antagonistic, with flow stagnation restraining the effect of sewage contamination, lessening the grazing activity.

How will biofilm microbial communities' proportions (bacteria, fungi and algae) change under the effects of flow stagnation and sewage contamination? The main community structure and diversity of stream biofilms reflected an overall effect of sewage contamination under water stagnation condition, which was affected by an additional grazing stressor (Chapter 5). Stressors interaction induced unequal responses among biofilm associated bacteria, fungi and algae. Interacting anthropogenic stressors (sewage × flow stagnation) effect on microbial diversity was found additive for algae and non-additive for the other biofilm assemblages exhibiting antagonistic effects for bacteria and synergistic for fungi. Accordingly, alterations in community

structure of biofilm assemblages induced by water contaminants and water scarcity effects have been previously detected (e.g. Pesce et al. 2016, Romero et al. 2018).

Are the anthropogenic stressors effects conditioned by a natural pressure (grazing activity)?
When adding a natural grazing pressure to flow stagnation and sewage contamination, it was observed an overall increase of fungi diversity, while algae and bacteria decreased, following the same pattern observed for the effect of anthropogenic stressors without the herbivory factor.

In fact, in Chapter 5 is possible to expose that grazing activity appears to stimulate algal abundance and diversity, in opposition to the decreased effect for the remaining biofilm assemblages, with particularly marked effects on fungi. Indeed, invertebrate feeding preference for fungi is already acknowledged (Arsuffi and Suberkropp 1989, Gessner et al. 2007, Graça et al. 2001). Given this, is plausible to consider that a moderate disturbance in biofilm driven by grazing activity might have boosted the settlement of new algae (probably more intrusive and tolerant), by also generating further micro-niches by grazing and removing the other biofilm assemblages, especially fungi. Additionally, such disturbance might have disrupted biofilm matrix releasing nutrients, which in turn might favour a higher algal proliferation (Battin et al. 2003, McCormick and Stevenson 1991).

Besides, triple stressors effect resulted in a shift of the microbial proportions within biofilm distributing them in a more even way, apportioning bacteria, fungi and algae in similar relative proportions. From a wider perspective, this may suggest that the effect of such co-occurring stressors has the potential to decrease the natural heterogeneity within stream ecosystems and may contribute to biota homogenization and ultimately, to biodiversity decline. Given the important role of stream biofilms in ecosystem functioning (e.g. primary production, nutrient recycling, carbon fixation) (Battin et al. 2016), it is reasonable to assume that by shifting their assemblages' proportions and relative composition will promote alterations in their key functions. Thus, variations in the structure and core functions of biofilms produced by microbial communities under the influence of stressors may affect the aquatic trophic chain and nutrient cycling (Barranguet et al 2005, Sabater et al. 2007, Woodward et al. 2012), eventually leading to the unbalancing of ecosystem processes defined by the single microbial communities.

7.1.3. Multiple stressors in the "real world" (Section IV)

How does hydrological and physicochemical alterations be reflected over time by biota structure and functions within a natural system impacted by multiple-stressors (i.e. urban

stream)? Streams in urban settings are constantly influenced by a higher number of multiple-stressors associated with stream hydrology, habitat, and water chemistry (Waite et al. 2019). So, assessing single stressors effects in such natural context is a huge challenge. Though the previous efforts (Chapters 2, 3, 4 and 5) to disentangle multiple-stressors effects on diverse biological receptors by employing factorial designs in mesocosms approaches was proven to be successful, by observing and recognising stressors impacts in a realistic scenario may also be enlightening and allows for a more integrative perspective that can help to understand the effects of disturbances at different scales.

In a case-study research, detailed in Chapter 6, was confirmed that a combination of several stressors was affecting stream macroinvertebrates community over time, resulting in overall low abundance and richness with the loss of more sensitive species (i.e. EPT taxa) and dominance of tolerant and resistant ones such as Chironomidae and Oligochaeta. Such findings are in agreements with previous studies (Feio et al. 2015, Suren and McMurtrie 2005, Walsh et al. 2001) also showing an impoverishment of riverine communities as an indicator of the "urban stream syndrome" (Walsh et al. 2005, Wenger et al. 2009).

Sampling occasions presenting the better biological quality were associated to an environmental stream pattern of lower nitrates concentrations, whereas the worst biological quality was found in sampling occasions associated with patterns of higher peak discharges, higher water conductivity and hardness. As previously exposed (Ledford et al. 2017, Paul and Meyer 2001), urbanization increases surface runoff due to the impervious surfaces, promote severe alterations in streams hydrology and water quality as result of higher contaminants entering the system from the surrounding environments. Moreover, with a functional approach of the community found in the study site, was possible to associate the worst water quality sampling occasions to higher proportions of generalist species, with generalized feeding strategies, bigger-size individuals adapted to low water velocity with preference for silt and sandy habitats, and able to produce resistance forms.

Also, despite observing variations of community structure and trait composition over time, it was not found a seasonal pattern (i.e. discriminating winter, from spring, or from summer), which reflects climate impacts marked by a year of atypical precipitation patterns and several hydromorphological and water quality stress gradient interacting. Such co-occurring factors determine the unpredictability associated with ecological functioning of urban streams when compared to more naturalized streams.

In this case study, also was argued the potential of higher levels of taxonomic resolution in the identification of less sensible macroinvertebrates normally disregarded in evaluating biological

quality in streams (i.e. Chironomidae genera) and biological traits as a suitable tool to detect changes under multiple-stressors in the quality of urban streams.

7.1.4. Management implications

Any non-additive interaction, regardless of whether it act synergistically or antagonistically, poses complex challenges to ecosystem managers. Generally, it is assumed that for additive stressors (operating independently, not influencing each other) the mitigation of any of the individual stressors will yield predictable benefits (Darling and Cote 2008) and the measures of management and/or restauration could be ranked according to their benefits for ecosystem functioning and correspondent services. Though, when stressors interact, the type of interaction needs to be considered for selecting the suitable measures and the correct quantity of measures to achieve a good ecological status (Schinegger et al. 2018).

In accordance with previous studies (e.g. Gieswein et al. 2017, Jackson et al. 2016), the stressors interaction found throughout this research were mainly additive or antagonist (overview in Table 7.1). Therefore, we assume that for additive interactions (Chapters 4 and 5) management actions focused on a single stressor should cause a positive outcome, whereas in ecosystems affected by antagonistic stressor interactions (Chapters 4 and 5), both stressors may need to be removed or moderated to promote any substantial ecological recovery. As supported by the overall main findings, in a multiple-stressor context, sewage inputs often represent the dominant stressor which should be mitigated before other stressors, because when a single stressor dominates, it is expected that the biological response may reach a threshold past which it will not decrease even in the presence of a second stressor. However, in situations where oxygen depletion and low flow velocity are causing synergistic effects (Chapter 2), mitigation/restoration efforts focusing in reducing a single stressor, especially hypoxia, may yield a greater overall benefit than predictable.

Consequences of water pollution and water availability on biota structure and functioning are expected to be stronger with severe periods of low flow and increasing water demand forecasted (IPCC 2014). Thus, water management in Mediterranean rivers under global change should not only focus on the preservation of natural flow regimes (Chapter 6), but more importantly, avoid severe hypoxia conditions caused by sewage inputs limiting effluent permits (Chapters 2, 3, 4 and 5). Water authorities should apply a dual rule on emission standards concerning wastewater discharges in water bodies. Indeed, water authorities should set a generalized rule considering the possibility of setting a temporary low emission standard

according to the river carrying capacity, as it may be the case under seasonal low flow discharge periods.

Also, some local efforts in restoration/mitigation may go through creating areas in stream with higher physical turbulence to improve water reaeration rates (Chapters 2 and 3).

In conclusion, the main results of the present research corroborate previous findings (e.g. Teichert et al. 2016) providing evidence that the largest management/restoration benefits are expected for mitigating water pollution, oxygen depletion and flow fluctuations.

7.2. Final Remarks

Overall, the research enclosed in this Thesis on water scarcity and water pollution combined effects on freshwater biota, have demonstrated that:

- When water scarcity is a prevailing condition, human-impacts causing stream hypoxia have a negative impact on macroinvertebrates community by amplifying organisms' avoidance and triggering drift behaviour, more than the single stressors effects (synergistic effect). This change in drift patterns has potentially strong ecological consequences to higher trophic levels.
- Also, stressors interaction can induce a loss of trait diversity in the macroinvertebrate communities, which may reduce the provision of ecosystem services and the ability of a stream to recover when normal situations are re-established. The use of some key trait categories would be a valuable contribute to detect specific alterations in the ecosystem while providing an indirect link to ecosystem functioning.
- When assessing stream biofilm and their consumers, it was found that flow stagnation has an overall inhibitory effect, while in the opposing way, sewage input reveals an increase of biological responses, suggesting that individually, sewage contamination determines a direct effect on the functional response of primary producers and an indirect effect upon primary consumers.
- In a stream biofilm community, in which the different assemblages are highly interlinked, the same multiple stressor combination can be unfavourable for some groups (e.g. decreased bacteria and algae diversity) or advantageous for another (e.g. increased fungi diversity). Overall, the combination of sewage contamination and water stagnation can lead to shifts in biofilms and consequently may interfere in ecosystem functions. Yet grazers seem to be able to restrict the increase of bacteria and algae

diversity and might be an important agent in balancing the ecosystem functioning in the presence of those pressures.

- In a case study on a Mediterranean urban stream was observed a variation on macroinvertebrate community structure and traits over time, but not translating a seasonal variation. An impoverished community with low diversity and high abundance of tolerant taxa and resistance traits was associated with multiple-stressors characteristic of urban ecosystems showing patterns of *in stream* higher nutrient concentrations, high levels of conductivity and hardness and strong hydrological variations with peak discharges.
- Also, bioassessment using Chironomidae genus represents a potential good tool to monitor the quality of impacted streams and follow their ecological recovery after restoration measures, especially in initial steps where other better-known sensitive taxa (usually assessed) remain absent.
- When considering water management decisions in Mediterranean systems under the impact of water scarcity and water pollution, the overall results of the present research indicates that more attention should be given to sewage contamination by prioritizing measures to restrict effluent permits together with an intensification of water cleaning treatments, particularly during seasonal low flow discharges.

7.3. Research limitations and future perspectives

This Thesis gathered considerable scientific evidences upon water scarcity and water pollution interacting effects on aquatic organisms. Although the effects of these stressors on stream biota and its implications on ecosystem functioning were not fully addressed in the present research, the results had provided new contribute to achieve a better understanding of the multiple-stressors problematic in Mediterranean stream ecosystems. Nonetheless, interacting stressors has not yet been completely understood and current knowledge is still mostly limited to a confined number of stressor combination (Jackson et al. 2016). Moreover, it seems not likely to identify the effect of every interaction on every organism´s physiology and every ecosystem function because the number of stressors, and their potential interaction are constantly growing and further prone to variations given global changes (IPCC 2012, 2014). Thus, throughout the thesis work development, new research issues have arisen giving opening to elaborate on future studies, either as a complement, or that go beyond presented knowledge. In fact, future

research using the different mesocosm facilities employed in this thesis, can comprise a multitude of different settings ranging from different organisms that can be tested, different physiological responses, using a wider range of stressors magnitude or using factorial designs with additional factors. In more detail, further works proposals can be focus on:

- Extend the work developed in Chapters 2 and 3 to a longer experiment at least encompassing a day/night period allowing to study stressors impacts taking into consideration macroinvertebrate drift behaviour diel activity patterns (Elliot 2005, Skinner 1985). Also investigate a lower gradient of flow velocity on drift behaviour, since it seemed likely that a low flow velocity threshold from which an active drift should be started has not been reached in our current study.
- Broadening biota responses assessed in Chapter 4, including other physiological responses, as for example, reproductive rates on macroinvertebrates and enzymatic activity on biofilm, given that stressors effects are always dependent of the receptor (Segner et al. 2014). Also, testing other grazer species, or further including more than one specie could generate other direct response to stressors and indirect response through competition on resources in a more realistic scenario. Such experiments should also be extended in time and including more replicates.
- Investigate the influence of temperature increases as an additional factor to the studied stressors, based in the climate changes predictions of global warming. Higher temperatures have the potential to interfere with several processes either at organism physiological level as ecosystem chemical functioning.
- Investigate further on biofilm microbial species on Chapter 5 by proceeding with an additional molecular identification. It was shown that biofilms assemblages change their relative proportions and diversity under the effect of flow depletion and sewage contaminations, but which are the specific species reflecting those changes? And can a given species be used as an indicator of these stressors' effects? Also, new insights can be provided by testing a biofilm community from a more pristine ecosystem given that the impacts that were found in Chapters 4 and 5 may be underestimated because the tested biofilm was sourced from a moderate impacted stream and their microbial assemblages might comprise species naturally more resistant and resilience to the stressors that were tested.
- Investigate longer environmental change patterns on macroinvertebrates in the urban stream by extending the monitoring assessment work undertaken in Chapter 6. Evaluating and understanding longer temporal patterns of multiple-stressors and which are the most important stressors affecting community is fundamental to support

effective further restoration measures and identify the better effective periods of intervention in this type of ecosystem.

Overall, further research based on microcosms, mesocosms and field studies that combined experimental assays with natural community studies, is crucial to understand the effects of stressors at different scales in an integrative assessment under interacting stressors.

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